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RESEARCH ON THE SYNTHESIS OF OXYGEN BY A PHYSICOCHEMICAL SYSTEM

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TRW Inc.

FEBRUARY 1969

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AEROSPACE MEDICAL RESEARCH LABORATORY
AEROSPACE MEDICAL DIVISION
AIR FORCE SYSTEMS COMMAND
WRIGHT-PATTERSON AIR FORCE BASE, OHIO

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FOREWORD

This research was conducted by the Department of Biosciences and Electrochemistry, TRW Systems Group, TRW Inc., One Space Park, Redondo Beach, California in fulfillment of Air Force Contract No. F33615-67-C-1506, under the direction of contract monitor R. E. Bennett, Life Support Division, Biomedical Laboratory* Aerospace Medical Research Laboratory, Wright-Patterson Air Force Base, Ohio. The studies were conducted in support of Project 6373, "Equipment for Life Support in Aerospace," Task 637302, "Respiratory Support Equipment."

The studies presented began in April 1967, and were concluded in February 1968. Principal contributors to this study include: E. C. Dale, R. J. Day, N. L. Gale, E. T. Seo, H. P. Silverman, and N. Weliky.

This technical report has been reviewed and is approved.

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*The Life Support Division and the Biomedical Laboratory were abolished during a reorganization in December 1968.

ABSTRACT

Where space, weight, and power limitations are of major importance, photosynthesis is an inefficient process for providing the energy for the production of food and oxygen. The assimilation of carbon dioxide into food materials by green plants requires two important factors commonly provided by the photosynthetic process, adenosine triphosphate (ATP), and reduced triphosphopyridine nucleotide (TPNH). We have demonstrated that oxygen as well as enzymatically active TPNH can be generated by an electrochemical system that employs the mediating agents: methyl viologen and ferredoxin-TPN-reductase. This system has been used successfully in the presence of spinach chloroplast extracts and has been shown to stimulate TPNH-dependent fixation of carbon dioxide. Further study is recommended to allow greater understanding and definition of the system and its components, and to explore the possibility of producing ATP within the electrochemical cell.

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List of Abbreviations

The following abbreviations were used:

ATP	Adenosine-5'-triphosphate
TPN ⁺	Triphosphopyridine nucleotide (nicotinamide-adenine dinucleotide phosphate)
TPNH	Reduced form of TPN ⁺
Tris	Tris(hydroxymethyl)aminomethane
G-1-P	Glucose-1-Phosphate
G-6-P	Glucose-6-Phosphate
HMP	Hexose monophosphate
FDP	Fructose-1,6-diphosphate
FTR	Ferredoxin-TPN-reductase
MV ⁺⁺	Methyl viologen dication
PGA	3-phosphoglyceric acid
PEP	2-phosphoenolpyruvic acid
Asp	Aspartic acid
Ala	Alanine
Gly	Glycine
Glyc	Glyceric acid
Ser	Serine
Pyr	Pyruvic acid
Lac	Lactic acid

SECTION I

INTRODUCTION

In man's quest to extend his influence beyond his natural habitat, he is forced to consider the problems connected with a long term survival in a hostile environment that does not normally support life. He must concern himself with recreating or preserving for the duration of his sojourn those segments of the terrestrial environment or their substitutes that he requires for survival. Chief among these essentials presently of concern to engineers attempting to maintain man in space are reliable sources of food, oxygen, and disposal or recycling of his metabolic wastes.

When one tries to engineer the photosynthetic process for a space-going vehicle, many deficiencies in the system become magnified — not the least of which are the difficulty of maintaining green plants or algae in space and the inefficiency of the photochemical processes. The immense surface areas required to illuminate sufficient chlorophyll-based photosynthetic food producing systems to provide for several people, makes it logical to inquire into alternative energy sources for the conversion of carbon dioxide into food and oxygen.

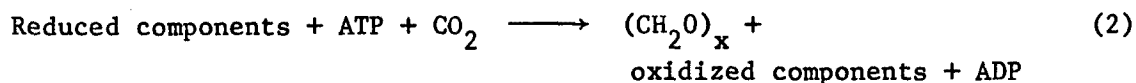
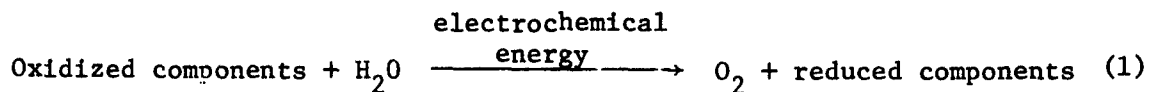
TRW Systems Group, therefore, has undertaken to study a system of carbon dioxide fixation that substitutes electrochemically activated steps for the normally photochemically activated steps. The present concept of using photosynthetic gas exchangers presents serious problems to the engineer designing life support systems for space vehicles. Photosynthesis is an inherently inefficient process, using only 2 percent to 3 percent of the incident white light and, where artificial lighting is required, this represents less than 1 percent of the incident energy. An electrochemically activated system promises to lead to the development of a gas exchanger that has a theoretical efficiency of 100 percent and a practical efficiency above 25 percent. Such a system does not require a light source, will operate at ambient temperatures and pressures, and will not require the large surface areas necessary for photosynthetic growth.

Our concept is to substitute electrical energy for light energy in the primary steps of chemical syntheses as they are performed in green plants. The process of photosynthesis as it is understood presently, involves the transfer of electrons between the components of the photosynthetic system resulting in the synthesis of active chemical reagents. This electron transfer is initiated by light energy. Electrical energy, supplied at an electrode, might reasonably be substituted for light energy in initiating electron transfer and, in an appropriate system, carbon dioxide fixation and reduction can be accomplished.

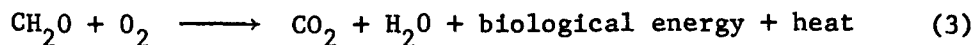
The feasibility of our approach was suggested by reports of TPNH synthesis using hydrogen gas, ferredoxin, and ferredoxin-TPN-reductase in the presence of hydrogenase (Shin and Arnon, 1965; Tagawa and Arnon, 1962; Trebst, Tsujimoto, and Arnon, 1958; Racker, 1955) and the fact that the hydrogen and ferredoxin were reported to reductively couple

carbon dioxide to acetyl-Coenzyme A to form pyruvic acid in cell-free extracts of photosynthetic bacteria (Buchanan, Bachofen, and Arnon, 1964).

Our approach uses electrolysis in an aqueous system to produce oxygen at the anode and TPNH in the cathode compartment. The electrochemical production of TPNH requires methyl viologen and ferredoxin-TPN-reductase as electron transfer agents. TPNH is then used in the cyclic fixation and reduction of carbon dioxide in situ to produce carbohydrates. The overall process may be summarized by the following equation:



where CH_2O represents carbohydrates that can be converted to other plant substances. The carbon dioxide and water required in the above processes may be the end products of carbohydrate metabolism in man:



The end result of this is the conversion of electrical energy to biological energy.

To be sure, other systems independent of artificial light are under investigation for application to long term space exploration. However, none of these systems offers the combined advantages of low temperature, high theoretical efficiency, the use of nonliving systems, and food synthesis. A brief theoretical comparison of electrochemo-synthesis with current algal gas exchangers operating on artificial light indicates that the TRW concept should result in a unit a fraction of the size, which will operate with a power requirement lower by about an order of magnitude.

SECTION II

ELECTROCHEMICAL REDUCTION OF PYRIDINE NUCLEOTIDES

BACKGROUND

The basis for the replacement of photosynthesis by electrolysis is the ability to electrochemically generate an enzymatically-active reduced triphosphopyridine nucleotide (TPNH). Thus, the chemical and electrochemical properties of unreduced triphosphopyridine nucleotide (TPN^+) are a very important consideration in this study.

TPN^+ and a closely related compound, diphosphopyridine nucleotide (DPN^+) are common oxidizing agents in biological systems. As a result, the nonenzymatic oxidation-reduction reactions of these pyridine nucleotides, in particular DPN^+ , have received considerable attention. The reactions of these substances in oxidation-reduction systems in free solution are very similar because the phosphate group that differentiates the two compounds is far from the portion of the molecule that undergoes reduction. Because of this close similarity in behavior, many studies have concentrated on the less expensive and more readily available DPN^+ . In general, the inferences drawn from such studies are equally applicable to TPN^+ .

Additional impetus for the study of pyridine nucleotides has been provided by the availability and suitability of 1-alkyl-3-carbamido-pyridinium salts as models for these compounds. The results and conclusions of the studies on pyridinium compounds have been reviewed and summarized by Kosower (1962). A salient feature of DPN^+ chemistry has been the ability of the pyridinium ring to react with nucleophiles to form dihydropyridine derivatives. Among the nucleophilic reducing agents investigated, dithionite ion reduction leads only to enzymatically active DPNH (a 1,4-dihydropyridine), whereas borohydride ion reduction leads to either a 1,2- or a 1,4-dihydropyridine configuration, depending on reaction conditions.

Convenient procedures for generating DPNH and TPNH that require a minimum of reagents and that do not require reagents that will interfere with subsequent reactions of the dinucleotides are difficult to find. However, the favorable standard reduction potential for the DPN^+/DPNH couple (obtained potentiometrically in the presence of enzyme) (Rodkey, 1955, 1959), -0.10 V vs nhe at 20°C (-0.31 V vs nhe or -0.55 V vs sce at $\text{pH} = 7$), suggests the use of electrochemical generation procedures. Several workers have attempted to prepare DPNH by controlled-potential electrolysis of DPN^+ (Ke, 1956; Powning and Kratzing, 1957; Kono, 1957; Kono and Nakamura, 1958). These attempts led to products with varying amounts of enzymatic activity. The early electrochemical literature concerning preparative and polarographic studies on the reduction of DPN^+ and related 3-carbamidopyridinium salts have been reviewed by Underwood and coworkers (Burnett and Underwood, 1965). Recent studies by Underwood and coworkers on 1-methyl-3-carbamidopyridinium chloride (Burnett and Underwood, 1965; Cunningham

and Underwood, 1967), and TPN^+ (Cunningham and Underwood, 1966, 1967) have been aimed at answering the several unresolved questions in the literature regarding the electrochemical reduction of DPN^+ . Briefly, Underwood's results indicate:

- The reduction patterns for all the 3-carbamidopyridinium compounds previously cited are essentially the same;
- The first of the two polarographic waves appears to represent a reversible, 1-electron transfer process; this is followed by an irreversible chemical step, the product of which is oxidized only at a much more positive potential;
- A mechanism involving free-radical formation followed by coupling (dimerization) of the radical through the 4-position is tenable;
- The 2-electron reduction product (1-alkyl-3-carbamido-1,4-dihydropyridine, DPNH , or TPNH) is formed on the second wave; with the 1-methyl compound, the 1,4-dihydropyridine is the only product, whereas with DPN^+ and TPN^+ a mixture of dihydropyridine (enzymatically active) and the coupled compound is obtained;
- The dihydro compound (authentic DPNH or TPNH) and the coupled product have different oxidation potentials;
- Fast-sweep cyclic chronoamperometry discloses the reversible reoxidation of a transient intermediate.

Other recent studies include a chronopotentiometric examination of the adsorption behavior of DPN^+ on mercury electrodes (Wilson and Epple, 1966), an investigation in which the instability of DPNH at pH values below 7 and the formation of the 1,2-dihydropyridine are suggested as the cause of enzymatic inactivity (Bergman, 1966), and one in which the electroreduction of the adenine ring of DPN^+ has been observed (Kotel'nikova and Solomatina, 1965).

MATERIALS AND METHODS

Apparatus

Polarographic measurements were made with a Sargent Model XV Polarograph equipped with a Sargent Model A IR Compensator for 3-electrode polarography. A dropping mercury electrode (dme), saturated calomel reference (sce), and platinum counter electrodes were used. The counter electrode was isolated from the working electrode (dme) compartment by a fritted-glass junction.

Controlled-potential electrolysis experiments were carried out with a Wenking Model 61RH potentiostat. The electrolysis current was recorded by either a Sargent MR or Varian G-14 recorder.

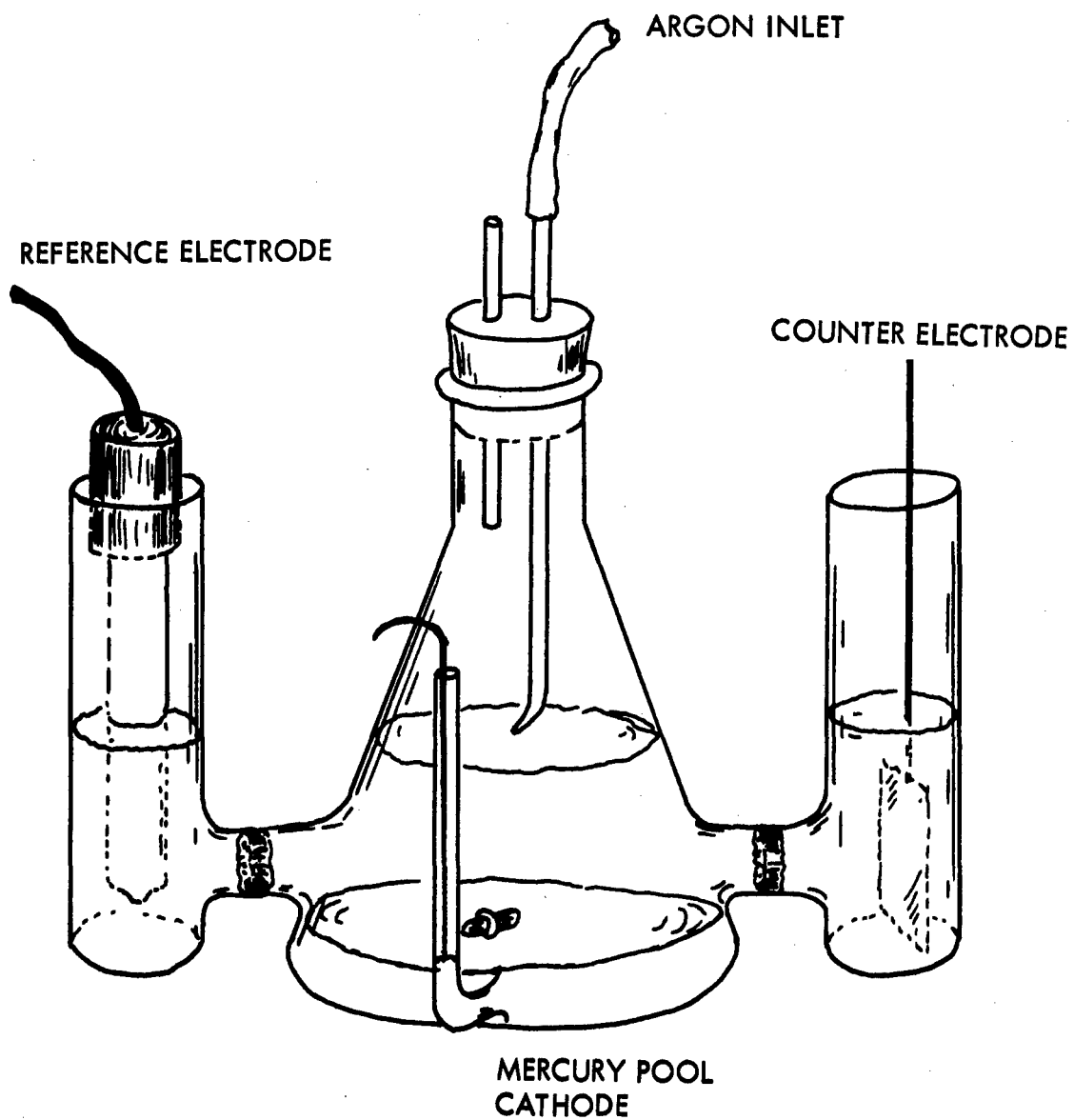


Figure 1. Diagram of electrolysis cell.

The electrolysis cell is diagramed in figure 1. The working electrode is a mercury pool contained in the center compartment, while the calomel reference and platinum counter electrodes are contained in the side compartments, with fritted-glass junctions between the compartments. The purpose of the conical shape of the working compartment was to decrease the ratio of solution volume to electrode area, and decrease the time required to complete an electrolysis. The solution in the working compartment was stirred with a magnetic stirring bar floating on the surface of the mercury pool.

Deaeration Procedures

In the case of the preliminary electrochemical studies, deaeration was accomplished in the conventional manner by bubbling water-saturated high-purity argon through the solutions. However, this procedure was unsatisfactory when extracts of plant materials were present in the solutions because severe foaming occurred. Thus, some form of deaeration that did not involve bubbling had to be used. The procedure adopted was to blow a rapid stream of argon across the surface of the solution while the solution was vigorously stirred. While oxygen removal by this technique was slower than by bubbling, a 30-minute deaeration period was sufficient to lower the oxygen concentration to a point where it did not significantly interfere with the electrochemical experiments.

Electrolysis Procedures

The electrolytic experiments were carried out at constant potential, with -1.1 vs sce being used for the direct reduction of TPN^+ or DPN^+ , while -0.8 V was used for the indirect process using methyl viologen. Integration of the current was accomplished graphically from the recorded current-time curve. Polarograms were taken periodically during the electrolysis by disconnecting the potentiostat, inserting the dme into the electrolysis cell, and using the same reference and counter electrodes for the polarographic measurements as were used for the potentiostat circuit. Typical concentrations in the solution prior to indirect electrolysis were 10^{-4} M methyl viologen, 5×10^{-4} M TPN^+ or DPN^+ , 0.5 mg/ml ferredoxin-TPN-reductase, 0.1 M tris(hydroxymethyl)aminomethane, pH 8.0.

Combined Electrolysis and Carbon Dioxide Fixation

The electrolytic cell for the combined experiments was set up in a manner similar to that used for the regular electrolysis studies. To decrease the chance of spurious effects, the solution used in the reference and counter electrode compartments was pure tris(hydroxymethyl)aminomethane buffer, pH 7.5, approximately 0.05 M, to correspond roughly with the ionic strength of the solution in the cathode compartment.

The reaction mixture within the cathode compartment was essentially the same as that described in a subsequent section for the carbon dioxide fixation study, with the following changes: methyl vio-

logen 8.0×10^{-5} M; ferredoxin-TPN-reductase (FTR), 0.5 mg/ml; TPN⁺, 8.0×10^{-4} M. The final solution volume in the cathode compartment was 20.0 ml. All reactants with the exception of the bicarbonate solution were placed within the cell and purged for 25 minutes with pure argon, after which, 0.8 ml of 0.1 M bicarbonate solution was added. The system was then purged for another 5 minutes and electrolysis begun. While it is possible some oxygen could have entered the cell during the addition of the bicarbonate, the amount remaining after the second purging would not be significant.

The electrolysis was interrupted at desired intervals, samples were withdrawn by pipette or syringe without stopping the continuous purge. A 5-minute interval was allowed for removal of traces of oxygen introduced by the sampling procedure, and electrolysis was continued.

Control samples were prepared in separate Erlenmeyer flasks and treated in an identical fashion except that they were not electrolyzed.

RESULTS AND DISCUSSION

Direct Reduction of Pyridine Nucleotides

Because of uncertainty over the nature of the products resulting from the direct electrochemical reduction of DPN⁺ and TPN⁺, the electrochemistry of these compounds was investigated. Tris(hydroxymethyl)aminomethane (tris) buffer, pH 7.5 or 8.0, was used as the supporting electrolyte for these studies because tris is the major constituent of the assay solution used in the carbon dioxide fixation studies to be described later.

Polarography of DPN⁺ or TPN⁺ in 0.1 M tris buffer at a dropping mercury electrode (dme) disclosed a single reduction step in both cases. The half-wave potential ($E_{1/2}$) was -0.90 V vs a saturated calomel electrode (sce) for DPN⁺ and -0.93 V vs sce for TPN⁺ (fig. 2). The decomposition of the supporting electrolyte began at approximately -1.5 V vs sce and hence did not permit the determination of the presence or absence of a second DPN⁺ or TPN⁺ reduction step reported by Underwood and coworkers to take place at -1.7 V vs sce, in a tetra-n-propylammonium carbonate solution (Burnett and Underwood, 1965b).

To obtain useable quantities of reduced nucleotide, controlled potential electrolyses of DPN⁺ were carried out in 0.1 M tris buffer at a mercury-pool electrode maintained at -1.1 V vs sce until the DPN⁺ was almost completely reduced. Polarography of this solution at a dropping mercury electrode (fig. 3) gave an oxidation step at -0.36 V vs sce for the reoxidation of the reduced DPN⁺. Spectrophotometric measurements of the solution showed the appearance of a sharp absorption band at 3400 Å, characteristic for the reduced diphosphopyridine nucleotide (DPNH).

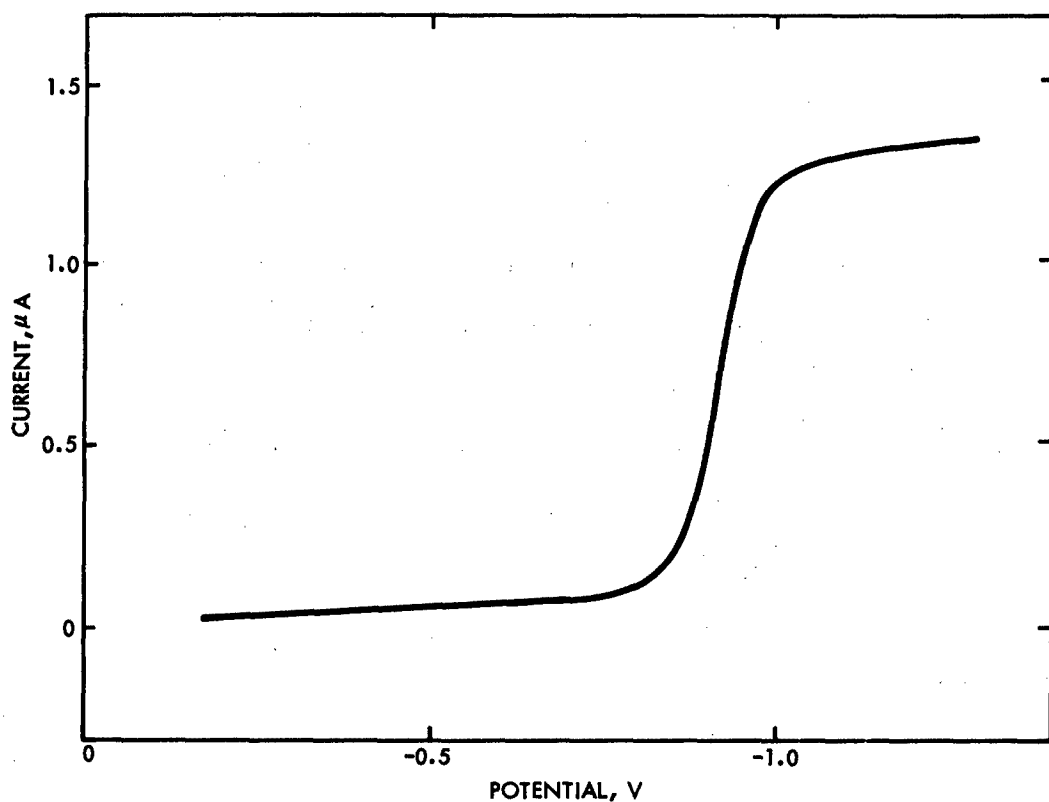


Figure 2. Envelope of the recorded polarogram of 5×10^{-4} M TPN^+ in 0.1 M tris buffer, pH 8.0.

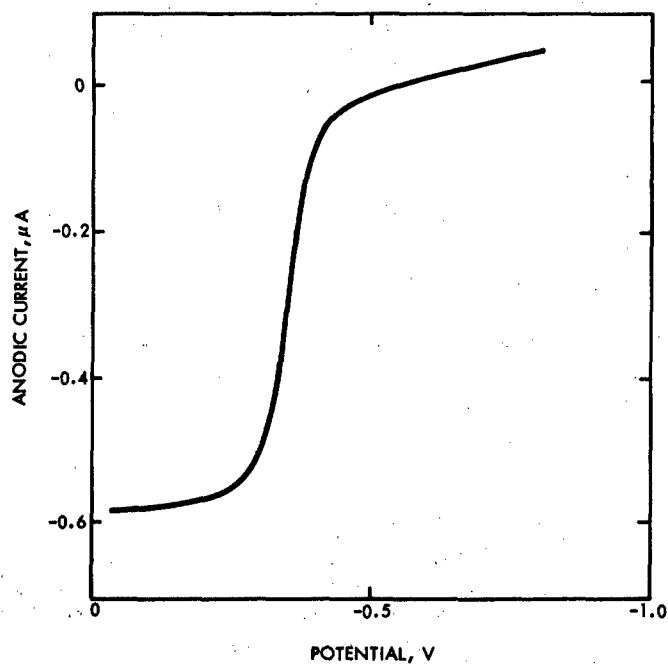


Figure 3. Envelope of the recorded polarogram of the product of the direct electrochemical reduction of DPN^+ in 0.1 M tris buffer, pH 8.0. Concentration of DPN^+ prior to electrolysis was 3×10^{-4} M.

For comparison, a solution of commercial DPNH (Sigma) also absorbed at 3400 Å, and the shape of the absorption peak was very similar to that of the direct electrolysis product of DPN^+ . Polarography of the DPNH solution, however, did not show any oxidation step that would correspond to the anodic wave which had been observed with electrolytically reduced DPN^+ . Finally, an enzymatic test confirmed the difference between the known DPNH and the DPN^+ direct electrolysis product. Using the standard DPNH enzymatic assay system, 2,6-dichlorophenolindophenol-diaphorase-DPNH, the commercial DPNH rapidly decolorized the indophenol, while the reduced DPN^+ (electrolysis product) had no observable effect on the 2,6-dichlorophenolindophenol.

These results are in agreement with those of Underwood and coworkers (Burnett and Underwood, 1965b; Cunningham and Underwood, 1966, 1967) who found that little, if any, DPNH or TPNH can be formed by the direct electrolysis of the nucleotides; instead, another substance is formed, tentatively identified as a coupled product (dimer) of the 1-electron reduction product. The dimer is not capable of acting as a reductant in enzymatic reactions in the manner of DPNH and TPNH. These results also agree with Underwood's findings that TPNH or DPNH cannot be differentiated from the corresponding electrolysis products by spectrophotometry, but can be differentiated by polarography or by enzymatic assay.

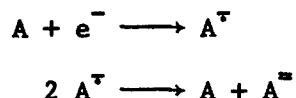
Indirect Reduction of Pyridine Nucleotides

Because the product of the direct electrolysis of DPN^+ or TPN^+ is not enzymatically active, it is not suited for use in the carbon dioxide fixation system. However, the possibility of indirect reduction was considered because the direct reduction of DPN^+ or TPN^+ at an electrode occurs at a potential significantly more cathodic than the theoretical thermodynamic potential for the reduction of DPN^+ or TPN^+ to DPNH or TPNH. Thus, the reversible $E_{1/2}$ for the conversion of DPN^+ to DPNH as determined potentiometrically by an enzyme system (Rodkey, 1955, 1959), is -0.59 V vs sce (pH 7.5), while the measured value for the reduction of DPN^+ to the inactive dimer is -0.90 V. The difference in potentials is such that if an additional system is added which could be reduced at the electrode at a potential less cathodic than that for the direct reduction of DPN^+ or TPN^+ , the reduction product of this added system might be capable of reducing TPN^+ (or DPN^+) to TPNH (or DPNH). An example of a system that might function in this manner is methyl viologen (chloride salt). This substance is reversibly reduced to the monocation radical with an $E_{1/2}$ of -0.68 V vs sce (Elofson and Edsberg, 1957), which is just negative to the DPN^+ /DPNH potential at pH 7.

Methyl viologen, in addition to having a suitable reduction potential, has several desirable properties for the present application. The reduction of methyl viologen is pH independent (Elofson and Edsberg, 1957) and its two 1-electron reduction steps are both reversible.*

* S. J. Kinsey and E. T. Seo, unpublished results, TRW Systems Group's Independent Research and Development Program.

Some complications involving adsorption and precipitation are associated with the second reduction process, as can be expected for the formation of a neutral organic species in aqueous solution, but the overall system is completely regenerative. The latter property of methyl viologen is significant because most organic compounds that form stable free radical species produce radical species that, in turn, disproportionate as follows:



The second reduction product, $A^{\cdot-}$, usually undergoes an irreversible chemical reaction with solvent or solution species, the net result being the depletion of the parent substance, A, (similar behavior is observed with oxidation processes) (Seo, Nelson, Marcoux, Leedy, and Adams, 1966; Nelson, Leedy, Seo, and Adams, 1967).

Since the potential for the reduction of methyl viologen to the cation radical is more cathodic than the potential for the production of TPNH or DPNH, reduced methyl viologen is theoretically capable of reducing DPN^+ or TPN^+ despite the fact that methyl viologen is reduced at a less cathodic potential than the potentials at which DPN^+ or TPN^+ are directly reduced at an electrode. Confirmation of this possibility is given by the fact that the rate of enzymatic reduction of DPN^+ to DPNH by hydrogenase, in the presence of ferredoxin and ferredoxin-TPN-reductase, is increased by the addition of methyl viologen (Shin and Arnon, 1965).

Because the indirect reduction of TPN^+ by reduced methyl viologen appeared to be the most feasible method of electrochemically preparing TPNH, three variations of this reaction were tested:

- direct reaction between DPN^+ or TPN^+ and methyl viologen cation radical
- enzymatic catalysis by diaphorase
- enzymatic catalysis by the ferredoxin-TPN-reductase system.

Of these, only the ferredoxin-TPN-reductase (FTR) system was capable of producing TPNH or DPNH at a significant rate. In the case of the other two variations, the rate of reaction was too slow to produce a useable amount of DPNH or TPNH during the course of a practical experiment.

Thus, methyl viologen can be reduced to the cation radical at -0.8 V vs sce in the presence of DPN^+ or TPN^+ without significant reduction of either of the nucleotides. If diaphorase is also present, there is a slight amount of reduction of the nucleotide, but the rate is very slow. However, if FTR is present, then DPN^+ or TPN^+ is rapidly reduced by the methyl viologen cation radical.

When controlled-potential electrolysis of methyl viologen is carried out at -0.8 V vs sce in the presence of FTR and excess TPN^+ or DPN^+ , the nucleotides react with the methyl viologen radical cation within a short period of time after the radical is formed. Hence, during most of the electrolysis, the methyl viologen concentration, and therefore the electrolysis current, remains approximately constant. Because the methyl viologen radical cation is intensely colored, the fact that there is no appreciable concentration of the radical until the latter stages of the experiment can be determined visually. Only near the end of the electrolysis where most of the nucleotide has been reduced does the current decrease, and the concentration of the reduced methyl viologen become appreciable. Also, polarograms were made during the course of the electrolysis which showed the decrease in the unreduced nucleotide concentration while the methyl viologen concentration remained approximately constant.

In order to determine the nature of the products of the indirect reduction of the nucleotides, the electrolysis solution was exposed to air at the completion of the experiment so that oxygen in the air could oxidize any cation radical present back to methyl viologen. Because the reduced nucleotides are not reoxidized by this procedure, tests to determine if TPNH or DPNH was present could be made without interference from other reductants.

The most critical test of the indirect reduction product is its capability to enzymatically reduce other materials. If a portion of the oxygenated solution was mixed with a 2,6-dichlorophenolindophenol solution, the indophenol was rapidly decolorized, indicating the presence of TPNH (or DPNH). The FTR present in the solution served in place of the usual diaphorase as the enzymatic catalyst.

After the electrolysis solution had been oxygenated to reoxidize the methyl viologen cation radical, it was deaerated again and the polarograms were made. These polarograms did not exhibit any oxidation step in the potential region where the inactive coupled product (dimer) would be oxidized if it were present. The lack of this anodic wave confirms the absence of the enzymatically inactive reduction product.

Integration of the electrolysis current, allowing for the residual current, and a correction for the amount of methyl viologen cation radical formed at the end of the electrolysis, gave a value for the TPNH concentration which agreed within experimental error with the TPNH concentration determined spectrophotometrically at 3400 \AA .

Thus, it is possible to produce TPNH or DPNH in good yield and with good current efficiency by an electrochemical system in which methyl viologen is first reduced at the electrode, and ferredoxin- TPN -reductase is used as the catalytic agent for the reduction of TPN^+ to TPNH by the resulting methyl viologen cation radical.

SECTION III

CARBON DIOXIDE FIXATION USING CELL-FREE CHLOROPLAST EXTRACTS

BACKGROUND

Photosynthetic Pathways in Green Plants

The fixation of carbon dioxide by autotrophic organisms is an intriguing and essential part of a continuous cycle of carbon compounds in nature. Organisms are defined as autotrophic or heterotrophic, depending on their independence of or dependence, respectively, on organic substances in their environment for growth. The carbohydrates, lipids, and proteins formed by autotrophic forms of life become the source of energy, vitamins, and building blocks essential for the growth of all heterotrophic forms of life, including man. Autotrophy is thus the master link between life and nonlife. The abundant supplies of energy found in sunlight are inaccessible to all except those autotrophs which are of the photosynthetic type: all of the heterotrophic animals and man are utterly dependent upon a continued flourishing of photosynthetic plants and the energy-rich organic compounds which they produce. Photosynthetic autotrophs such as green plants absorb carbon dioxide and produce food and oxygen. This process is, in effect, the reverse of the metabolic processes of heterotrophs, such as man, whereby food and oxygen are absorbed to yield energy and waste products, including carbon dioxide. This natural recycling of elements and compounds in our terrestrial environment is the result of a routine, yet exceedingly complex set of phenomena.

In any approach that attempts to substitute for a part of a natural biological system, some idea must be had concerning the components and mechanisms of the system. It is no reflection on the investigators in the field to note that all points have not yet been cleared up and that there are alternative working hypotheses for some of the steps. A realization of the importance of these schemes is helpful in evaluating alternative ways of approaching new problems that arise. From the work of investigators such as van Neil, Calvin, Varnon, San Pietro, Arnon, Jagendorf and others, schemes representative of different schools of thought and areas of investigation have been assembled and are presented in the Appendix.

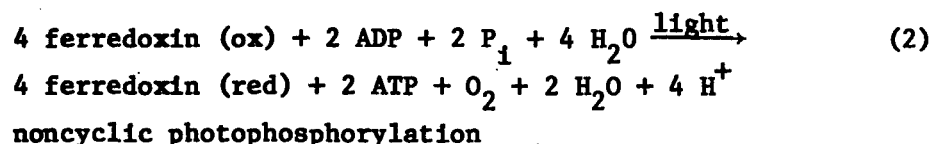
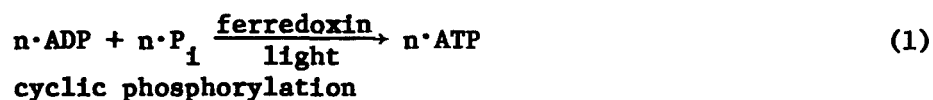
The Mechanism of Green Plant Photosynthesis

The photosynthetic process can be divided into a photochemical or light phase and a chemosynthetic or dark phase. Exposure of chlorophyll to visible light results in the photochemical reduction of triphosphopyridine nucleotide (TPN⁺) to TPNH and the concomitant formation of adenosine triphosphate (ATP). These compounds are subsequently utilized in a series of reactions which occur without additional light energy and which result in the chemical fixation of carbon dioxide into sugars and other components of the plant. Many reviews of this subject have been written (Arnon, 1965; Bishop, 1966; Calvin, 1962;

Clayton, 1963; Clayton, 1965; Hoch and Kok, 1961; Krasnovsky, 1960; Robinson, 1964; San Pietro and Black, 1965; Smith and French, 1963; van Neil, 1962; Vernon and Avron, 1965; Warburg, 1957).

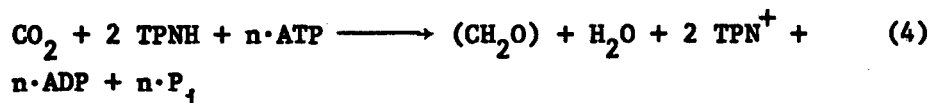
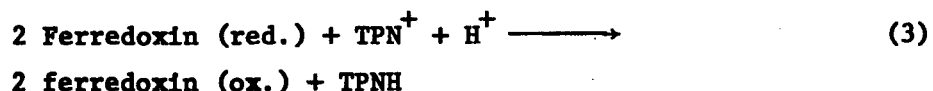
In the photochemical process, chlorophyll absorbs light energy and the resultant electronically excited molecule activates the reduction of an iron-containing protein, ferredoxin. The papers of Arnon and coworkers (Shin and Arnon, 1965; Tagawa and Arnon, 1962; Trebst, Tsujimoto, and Arnon, 1958; Arnon, 1965; Arnon, 1959; Arnon, Tsujimoto, and McSwain, 1965; Losada, Whatley, and Arnon, 1961; Arnon, Tsujimoto, and McSwain, 1967) differentiate the process into cyclic and noncyclic systems. The cyclic system is activated by low energy light and results only in the formation of ATP. The noncyclic system, activated by higher energy light, results in the synthesis of oxygen, a smaller quantity of ATP, and all the reduced enzymatic cofactors, such as TPNH, required for the incorporation of carbon dioxide into plant materials.

The net reactions of the light phase of photosynthesis, according to Arnon, are:



where ADP and P_1 are adenosine diphosphate and inorganic phosphate, respectively.

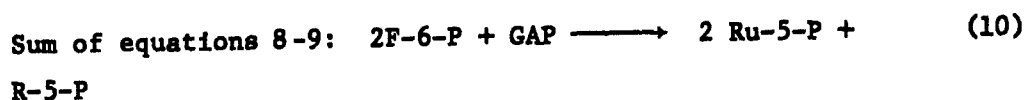
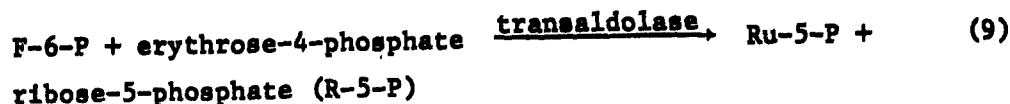
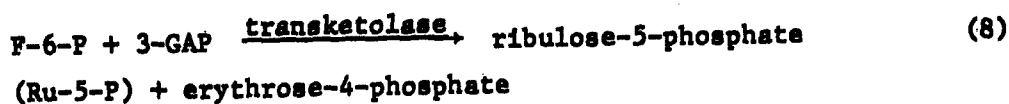
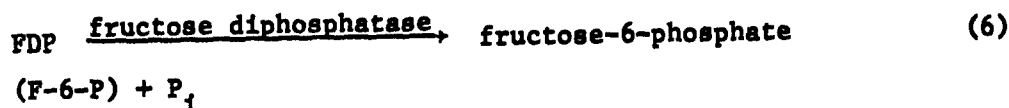
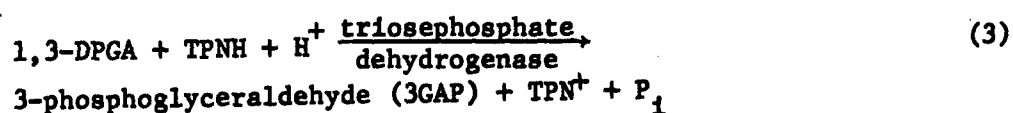
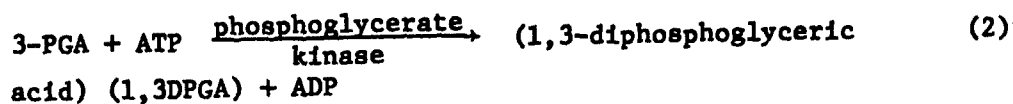
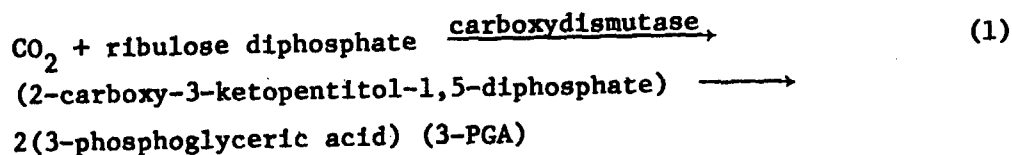
The reduced ferredoxin then reduces TPN^+ , which, along with ATP, is required for carbon dioxide fixation into plant materials.



A more detailed stepwise reaction sequence representing electron-hydrogen transport but omitting phosphorylation has been presented by Robinson (1964).

The most distinctive step of the dark phase of photosynthesis is the incorporation of carbon dioxide into the Calvin cycle (Calvin, 1962). Carbon dioxide, in the presence of carboxydismutase, reacts with ribulose-1,5-diphosphate to form a six-carbon intermediate which decomposes into 2 moles of 3-phosphoglyceric acid. The 3-phosphoglyceric acid goes

through a number of steps during which part of the carbon is drained off into carbohydrate, protein and lipid synthesis, and the synthesis of other plant components. The remainder is then reconverted to ribulose-1,5-diphosphate:



The concentrations and activities of the component enzymes and cofactors play a role in determining the steady state concentrations of the plant intermediates and plant materials. In experimental systems, the activities and concentrations of components are altered by the loss of metallic ions, enzymes, and other required substances through washing, physical damage, oxidation, and other environmental changes.

Reconstitution of disrupted cell systems to reproduce functions of the intact cell will require a great deal of study and care.

The sequence of reactions from which ATP and reduced pyridine nucleotides result, necessary for the reduction of carbon dioxide, is not yet resolved or agreed upon (Arnon, 1965; Bishop, 1966; Calvin, 1962; Clayton, 1963; Clayton, 1965; Hoch and Kok, 1961; Krasnovsky, 1960; Robinson, 1964; San Pietro and Black, 1965; Smith and French, 1963, Van Neil, 1962; Vernon and Avron, 1965; Warburg, 1957; Avron, Krogmann, and Jagendorf, 1958; Hind and Jagendorf, 1963). Arnon and coworkers (Arnon, 1965; Arnon, Tsujimoto, and McSwain, 1965) have suggested that the electron transport chain includes:

ferredoxin—ferredoxin-TPN—reductase—TPNH

Electron Acceptors and Inhibitors for Photosynthetic Reactions

Sufficient information has been obtained so that there is an extensive source of literature reporting experiments that have been successfully completed using substitutes for components of photo-phosphorylation and for the study of reaction inhibition in this system. The interpretation of the effects of these substances depends on the reaction scheme adopted.

The Hill reaction is the intercession of non-physiological electron acceptors, such as ferricyanide, benzoquinone, and some dyes, in reaction sequences where ferredoxin is the normal electron transfer agent. Other substances, such as nitrate, nitrite, viologen dyes, indophenols, phenazine methosulfate, flavin mononucleotide, N,N,N',N'-tetramethyl-p-phenylenedimine, toluylene blue, thionine, and methylene blue are also reduced at other steps of the photosynthetic reaction sequence. The steps in which it is believed that some of these substances participate are indicated in the Vernon and Avron (1965) and the Arnon, Tsujimoto, and McSwain (1965) formulations.

Bypassing the Photochemical Step in Photosynthesis

From the mechanisms shown in figures 1-14 of the Appendix, the reduction of either ferredoxin, ferredoxin-TPN-reductase, triphosphopyridine nucleotide, or diphosphopyridine nucleotide and the addition of adenosine triphosphate to disrupted plant cells should be sufficient to permit carbon dioxide fixation in the absence of light.

Carbon dioxide fixation has been demonstrated in the dark by many investigators. Algae of the species Scenedesmus, Rhaphidum, Ankistrodemes, and others can adapt to reduce carbon dioxide without exposure to light (Bishop, 1966; Gaffron, 1944). These algae have normal photosynthetic systems. In the dark, they produce an hydrogenase which permits the use of hydrogen to reduce the primary component of the electron transfer chain so that energy is available for the reductive synthesis of plant components. The cell components produced do not differ from the photosynthetic components. These organisms are able to bypass the photochemical step and substitute for it a reductive step

which makes available to the electron transfer system a reduced substance, presumably ferredoxin, which serves to initiate the synthesis of ATP and the reduction of pyridine nucleotides which are then used in the synthesis of plant structural components and metabolism.

Several studies in cell-free systems have demonstrated that excited chlorophyll provides the primary reducing factor in the electron transport chain (Arnon, 1965; Bishop, 1966; and Calvin, 1962).

Racker (1955) demonstrated that spinach fractions, free of cells and chlorophyll, synthesized hexose monophosphate in the dark if ATP, reduced DPN^+ and a system to keep the DPN^+ reduced (either alcohol and alcohol dehydrogenase or hydrogen and a diphosphopyridine nucleotide linked hydrogenase) were present.

Trebst, Tsujimoto, and Arnon (1958) reported carbon dioxide fixation in the dark in chlorophyll-free systems if ATP is provided and if either TPNH is added in substrate amounts or if TPNH is added in catalytic amounts and the appropriate dehydrogenase is used in conjunction with glucose-6-phosphate, 6-phosphogluconate, or isocitrate to effect reduction of TPNH oxidized in the fixation process.

Tagawa and Arnon (1962) demonstrated that ferredoxin could be reduced in the dark by hydrogen in the presence of a bacterial hydrogenase. They further showed that if the hydrogenase were added, hydrogen could reduce TPN^+ in the presence of broken chloroplasts. The minimum requirements were ferredoxin and a flavoprotein fraction of leaves. No TPN^+ reduction was observed if the flavoprotein fraction was omitted.

Shin and Arnon (1965) used a system of purified components: hydrogen, hydrogenase, spinach ferredoxin, ferredoxin-TPN-reductase, and TPN^+ . The TPN^+ was reduced only if the reductase was present. Addition of a viologen dye markedly increased the rate at which TPN^+ was reduced. The interpretation was that the hydrogenase catalyzed electron transfer from the hydrogen to the viologen dyes faster than from hydrogen to ferredoxin, and that reduced viologen dyes reduce ferredoxin non-enzymatically.

In summary, through careful and well planned experiments, the incorporation of carbon dioxide into carbohydrates has been demonstrated by (a) supplying reduced TPN^+ or ferredoxin-TPN-reductase, (b) by providing a mechanism through which TPN^+ , ferredoxin-TPN-reductase, or ferredoxin can be reduced in intact cells, disrupted cells, or cell fractions.

In the two cases where carbon dioxide fixation was directly observed, ATP was added to the medium. It is still questionable, therefore, that sufficient ATP can be generated by any of these alternatives to photoreduction, to support carbon dioxide fixation.

We have been able to reduce TPN^+ electrochemically to TPNH by making use of ferredoxin, the enzyme ferredoxin- TPN -reductase, and methyl viologen as transfer agents. Using this system, we have been able to demonstrate the utilization of the resultant TPNH as a reducing agent in initial enzymatic processes that are required to incorporate carbon dioxide into plant materials.

MATERIALS AND METHODS

Source of Spinach and Preparation of Chloroplast Extract

Spinach was obtained from local markets within 24 hours after harvest. Once received in the laboratory, all operations in the preparation of extract were done in a refrigerated laboratory at 4-6 C.

The isolation and disruption of chloroplasts were carried out according to the method described by Tagawa and Arnon (1962). After initial selection of fresh, young spinach leaves, the stalk and midrib were cut out and discarded. The leaves were then washed thoroughly in cold distilled water and drained for 5-10 minutes on paper towels. Approximately 300 to 400 g of washed leaves were minced with scissors and packed into a Waring blender. Twenty ml of 0.2 M tris (hydroxymethyl)aminomethane (tris) buffer, pH 8.0, and 200 ml of 0.35 M NaCl were added, and the suspension was ground for 15-30 sec. The resulting thick suspension was squeezed through two layers of fine cheesecloth to remove debris. The chloroplasts were removed from the suspension by centrifugation at $7000 \times g$ for 10 minutes in a Servall Model RC-2 refrigerated centrifuge at 0-5 C. The pellets were resuspended in a minimum volume of cold redistilled water, and following determination of chlorophyll, the volume was adjusted so as to give a chlorophyll concentration of 2 mg per ml. The resulting hypotonic suspension was stirred in the cold for 30 min to allow lysis of the chloroplasts.

An alternative method for the disruption of the chloroplasts involved the treatment of the diluted suspension with the maximum output of the Branson Model S-125 Sonifier for three periods of 1 minute each, alternated with 1 minute of cooling in a bath of ice and salt water. No significant differences in behavior of the extracts prepared by these two methods were apparent, although the chlorophyll content of the extracts prepared by ultrasonic vibration was generally higher.

Following lysis or sonic treatment, whole chloroplasts and debris were removed by centrifugation for 30 min at $35,000 \times g$, and the resulting green, transparent chloroplast-free extract was decanted and used immediately for carbon dioxide fixation studies. Approximately 50 ml of extract were usually obtained from 350 g of spinach leaves.

Estimation of Chlorophyll

Chlorophyll content was measured by the method of Arnon (1949). A 0.1 ml sample of chloroplast suspension was diluted to 25 ml with 80 percent acetone in water in a low-actinic volumetric flask, so as to exclude light. The suspension was shaken vigorously to allow total extraction of chlorophyll. The resulting solution was then filtered through a Solvinert filter (Millipore) with a glass fiber prefilter mounted in a Swinney stainless-steel filter holder. The filtered chlorophyll extract was placed in a capped 1-cm cuvette for spectrophotometric analysis. Measurements were made against a blank of 80 percent acetone in water. The concentration of chlorophyll was estimated by the following relationship:

$$[A_{6630}(8.02) + A_{6450}(20.2)] \times 250 = \text{chlorophyll concentration in mg/liter.}$$

Preparation of Ferredoxin-TPN-reductase

Ferredoxin-TPN-reductase (FTR) was prepared in the cold from fresh whole spinach leaves by the procedure of Shin, Tagawa, and Arnon (1963). One hundred grams of washed leaves were blended with 150 ml of cold redistilled water for 2 minutes at maximum speed in a Waring blender. The mixture was strained through two layers of cheesecloth, centrifuged for 15 min at $12,000 \times g$, and the pellet discarded. The pH of the supernatant fluid was adjusted to 7.5 by the addition of 1 M tris. Acetone was added slowly to the extract to a concentration of 35 percent. The precipitate was removed by centrifugation for 5 min at $1,000 \times g$ and discarded. The concentration of acetone was slowly raised to 75 percent, and the resulting precipitate, containing the desired enzyme activity was removed by centrifugation for 5 min at $1,000 \times g$. The supernatant liquid was discarded. The precipitated protein was redissolved in 10 ml of 0.005 M tris, pH 8.0. The solution was centrifuged for 20 minutes at $18,000 \times g$, and the pellet discarded. The supernatant solution was dialyzed against 2 liters of 0.005 M tris at pH 8. The bath was changed twice during the dialysis procedure. The dialyzed enzyme preparation was then shell frozen, lyophilized, and stored below 0 C under desiccation.

Measurement of Carbon Dioxide Fixation

Basic reaction mixture.

The basic reaction mixture (2.5-5.0 ml) consisted of: Chloroplast extract equivalent to 0.80 mg chlorophyll/ml; tris(hydroxymethyl)aminomethane buffer, 3.20×10^{-2} M; $MgCl_2$, 2.00×10^{-3} M; $MnCl_2$, 8.00×10^{-4} M; Na_2HPO_4 , 2.00×10^{-3} M; ATP, 8.00×10^{-4} M; $[^{14}C]$ -sodium bicarbonate, 4.00×10^{-3} M. The following reducing compounds were present in most reaction mixtures: ascorbic acid, 4.00×10^{-3} M; L-cysteine-HCl, 6.00×10^{-4} M; glycose-1-phosphate, 1.2×10^{-4} M. All components were brought to pH 7.5 with HCl after dissolving. In many cases, R-5-P was added (final conc. 8×10^{-4} M) to increase the amount of carbon dioxide fixed. Reactants were placed in test tubes or 10 ml Erlenmeyer

flasks. In those experiments in which argon atmospheres were used, the flasks were either (a) flushed for 30 sec with ultra-pure-grade argon (Air Products) and stoppered, or (b) fitted with a two-hole stopper with argon delivered continuously to the surface of the liquid through a Pasteur pipette. Stoppered flasks were held at 22 C and agitated using a reciprocal shaker or a magnetic stirring bar.

In experiments involving electrolysis, where preliminary deaeration of all reactants with the exception of radioactive bicarbonate was necessary, control samples were treated in the same fashion and the reaction begun by the addition of bicarbonate to all vessels.

For those extracts which contained considerable amounts of residual chlorophyll, the entire reaction was conducted in a darkened room.

Radioactivity Determination.

Aliquots of 0.1 ml were removed from each reaction mixture at various times during the experiment. These samples were placed in stainless steel planchettes and 0.2 ml 4 N HCl was added to remove excess "non-fixed" bicarbonate. Two-tenths ml of acetone was added to aid in uniform spreading of the samples on the bottom of the planchette, and the samples were dried under an infrared heat lamp. Radioactivity was determined using a Nuclear Chicago automatic planchette counter.

Analysis of Products of Carbon Dioxide Fixation

Preparation of Sample

At the end of each CO₂ fixation experiment, the reaction was stopped by the addition to all reaction vessels of perchloric acid to a final concentration of 7 percent. The mixtures were then neutralized to pH 7 with 5 N potassium hydroxide, and held overnight in the refrigerator (2-4 C). The precipitated protein and potassium perchlorate were discarded and the supernatant, containing the products of carbon dioxide fixation, was analyzed by paper chromatographic methods.

Paper Chromatography

Samples were spotted (approx. 0.1 ml) on Whatman 1 Chromatographic papers (18 x 22") and submitted to descending two-dimensional chromatographic separation. The solvent systems employed were those described by Tyszkiewicz (1962) and consisted of: I. isobutyric acid:1 N ammonium hydroxide:0.1 M ethylenediaminetetraacetic acid (EDTA) in the ratios (v/v) 100:60:1.6. II. n-butanol:propionic acid:water in the ratios 375:180:245. Approximately 24 hours were required for development in the first solvent, after which the papers were air dried. The second solvent was allowed to flow for approximately 20 hours at right angles to the direction of flow of the first solvent. After drying, spots were detected using ultra violet absorption, specific spray reagents, and/or by radioautography. Identification was by comparison with the behavior of known compounds.

Spray Reagents

The spray reagents employed were described by Block, Durum, and Zweig (1955). Ninhydrin (0.3 percent) in 95 percent ethanol was used for detection of amino acids and other amine compounds. Solutions of (1) aniline and phthalic acid in water-saturated n-butanol and (2) ammoniacal silver nitrate were used for detecting sugars, sugar phosphates, and organic acids.

Radioautography

The developed chromatograms were trimmed to fit 14" x 17" film cassettes, and placed in direct contact with Kodak blue sensitive X-ray film for periods of 3-12 days.

Determination of Distribution of Radioactivity

After location of radioactive compounds by radioautography, the spots were cut out, placed in separate scintillation vials, and eluted with 0.5 ml of 0.1 N HCl. The paper was left at the bottom of the vial. Fourteen ml of a three-phase scintillation cocktail were added, made from: 500 ml toluene, 500 ml p-dioxane, 300 ml ethanol, 65 g naphthalene, 6.4 g PPO, 1.0 ml Spectrafluor. The vials were then shaken vigorously for 30 min. The radioactivity of each spot was determined using a Nuclear Chicago liquid scintillation spectrometer.

Protein Determination

Protein content of chloroplast-free extracts was estimated by comparing the absorbancies at 2600 and 2800 Å according to the method of Warburg and Christian (1941).

Spectrophotometric Determinations

Spectrophotometric determinations were made using a Cary Model 15 recording spectrophotometer.

Special Chemicals

[¹⁴C]-labeled glucose-6-phosphate, [¹⁴C]-3-phosphoglyceric acid, [¹⁴C]-glyceric acid, and [¹⁴C]-sodium bicarbonate were obtained from Calbiochem, Los Angeles. Adenosine-5'-triphosphate, as the disodium salt was also obtained from Calbiochem. Triphosphopyridine nucleotide, oxidized and reduced forms, glucose-1-phosphate, tris(hydroxymethyl)-aminomethane, and ribose-5-phosphate were obtained from Sigma, St. Louis. Methyl viologen was a product of Mann Research Chemicals, New York.

RESULTS AND DISCUSSION

Cell-free Carbon Dioxide Incorporation System Using Chemical Reduction

Before attempting to combine the electrochemical reduction of TPN^+ and the utilization of the product (TPNH) by cell-free extracts, it was necessary to establish that the methods and results described by Trebst, Tsujimoto, and Arnon (1958) could be repeated in our laboratory. Arnon and his associates found that the incorporation of carbon dioxide by extracts of spinach chloroplasts was stimulated by the addition of TPNH. The increased amounts of CO_2 incorporated were presumably a result of the production of labeled sugar mono- and diphosphates, which are known to require TPNH for their formation. Thus, the carbon dioxide fixing ability of chloroplast extracts appeared to be the ideal system for detecting the presence of electrochemically generated TPNH and its utilization for the synthesis of plant materials.

Chloroplast extracts, prepared as described under Methods generally contained 10-20 mg of protein per ml, and were active in fixing carbon dioxide.

The results of experiments duplicating those of Trebst, *et al.* are shown in table I. The inclusion of TPNH was found to increase the amount of carbon dioxide fixed by a factor of almost 3 over the control which contained no TPNH. Incorporation of carbon dioxide was measured by the radioactivity of the nonvolatile portion of the acidified test solution, using ^{14}C -bicarbonate as the source of carbon dioxide.

Table I. Duplication of Results of Trebst, Tsujimoto, and Arnon (1958).

Additions	<u>Carbon Dioxide Incorporated</u> counts per min. per ml at 30 min.
1. None	820
2. ATP	1820
3. ATP + TPNH	5200

The basic reaction mixture contained the following at pH 7.5: chloroplast extract equivalent to 0.8 mg chlorophyll/ml; 3.2×10^{-2} M tris; 2×10^{-3} M MgCl_2 ; 8×10^{-4} M MnCl_2 ; 2×10^{-3} M Na_2HPO_4 ; 4×10^{-3} M ascorbic acid; 6×10^{-4} M cysteine-HCl; 1.2×10^{-4} M G-1-P; 4×10^{-3} M ^{14}C -sodium bicarbonate. The following additions were made: 1. none; 2. 8×10^{-4} M ATP; 3. 8×10^{-4} M TPNH + 8×10^{-4} M ATP. Specific activity of ^{14}C -sodium bicarbonate was 4×10^4 cpm/ μmole . Final volume was 2.5 ml.

In addition to the components Trebst, et al. found necessary for carbon dioxide assimilation using chemical reduction (TPNH), electrochemical reduction required methyl viologen as an electron transfer agent. The results of a preliminary experiment to determine the effects of methyl viologen dication on a system in which carbon dioxide fixation was occurring are presented in table II.

The inclusion of methyl viologen dication caused a significant increase in the amount of carbon dioxide assimilated, and pointed out the need for further study of the effects of this compound on the carbon dioxide fixing system.

Table II. Effect of Oxidized Methyl Viologen on CO₂-fixing System.

Additions	Carbon Dioxide Incorporated	
	counts per min. per ml after	
	15 min.	30 min.
1. ATP	383	704
2. ATP + TPNH	2570	4700
3. ATP + methyl viologen (ox.) + TPNH	4180	8040
4. None	205	339

The basic reaction mixture was the same as that described in table I. The following additions were made: 1. 8×10^{-4} M ATP; 2. 8×10^{-4} M ATP + 8×10^{-4} M TPNH; 3. 8×10^{-4} M ATP + 8×10^{-5} M mv^{++} + 8×10^{-4} M TPNH. 4. none. Specific activity of [¹⁴C]-sodium bicarbonate was 4×10^4 cpm/ μ mole. Final volume was 2.5 ml.

If TPNH was excluded (table III), methyl viologen dication had little or no effect on the amount of carbon dioxide fixed by the chloroplast extract. This conclusion was confirmed by the results shown in figure 4. The inclusion of methyl viologen dication increased incorporation of carbon dioxide if TPNH was present but slightly decreased incorporation if TPNH⁺ was present. The presence of ribose-5-phosphate increased total incorporation but did not change the relative effect of methyl viologen. Doubling the concentration of ATP increased carbon dioxide incorporation by approximately 7.5 percent (see curve f of figure 4). However, small changes in ATP concentrations had little effect on the amount or rate of carbon dioxide fixation.

These results demonstrated that the stimulatory effect of TPNH on CO₂ incorporation could be observed in the presence of methyl viologen dication, ribose-5-phosphate (R-5-P), and ATP.

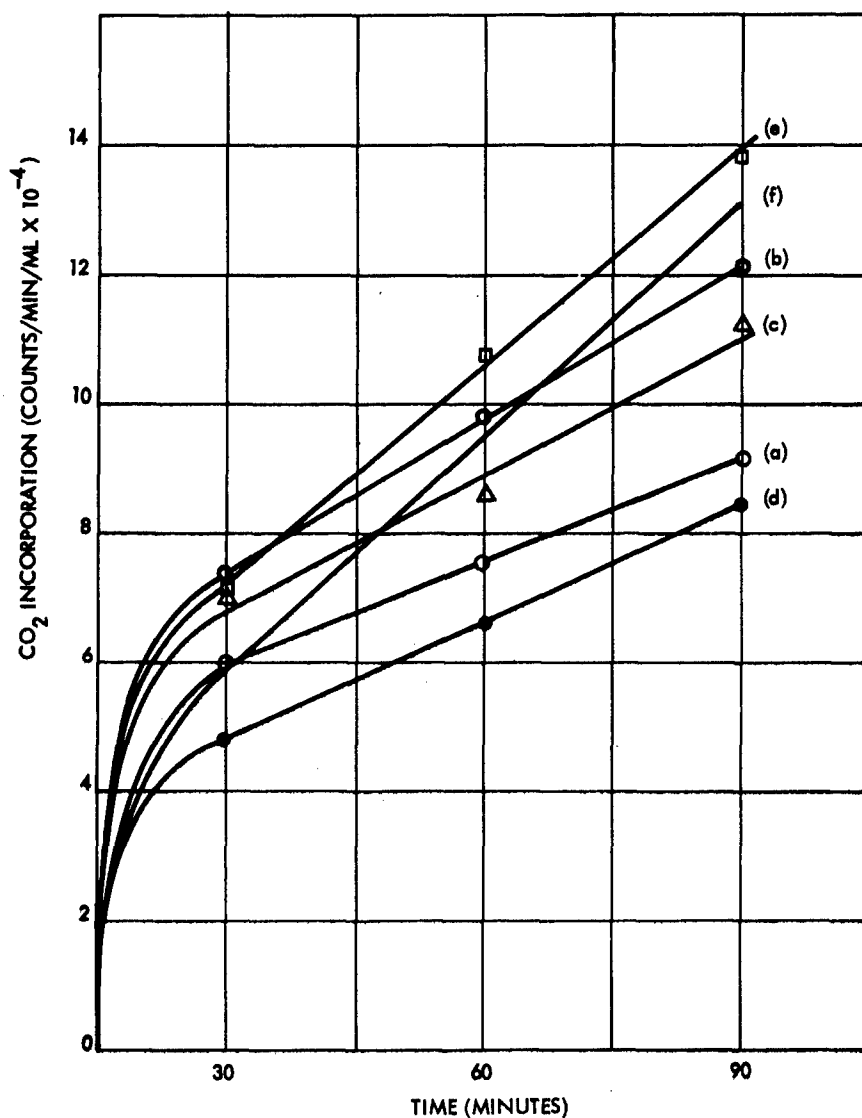


Figure 4. Rate of CO_2 Fixation with Varying Concentrations of ATP and Methyl Viologen. Conditions were as listed for the basic reaction mixture in table I, with the following additions: a. $8 \times 10^{-4} \text{ M TPN}^+ + 8 \times 10^{-4} \text{ M ATP}$; b. $8 \times 10^{-4} \text{ M TPNH} + 8 \times 10^{-4} \text{ M ATP}$; c. $8 \times 10^{-4} \text{ M mv}^{++}$; e. $8 \times 10^{-4} \text{ M TPNH} + 8 \times 10^{-5} \text{ M mv}^{++} + 8 \times 10^{-4} \text{ M ATP}$; f. $8 \times 10^{-4} \text{ M TPNH} + 1.6 \times 10^{-3} \text{ M ATP}$. Specific activity of $[^{14}\text{C}]$ -sodium bicarbonate was $3.2 \times 10^5 \text{ cpm}/\mu\text{mole}$.

Table III. Effect of Oxidized Methyl Viologen on CO₂-fixing System.

Additions	Carbon Dioxide Incorporated counts per min per ml at 30 min.
1. None	215
2. ATP	934
3. ATP + methyl viologen	852
4. methyl viologen	320

The basic reaction mixture was the same as that described in table I. Additions were as follows: 1. None. 2. 8×10^{-4} M ATP; 3. 8×10^{-4} M ATP + 8×10^{-5} M mv⁺⁺; 4. 8×10^{-5} M mv⁺⁺. Specific activity of [¹⁴C]-sodium bicarbonate was 4×10^4 cpm/ μ mole. Final volume was 2.5 ml.

In the presence of added R-5-P, however, the amount of ribulose-1,5-diphosphate (RuDP) produced from the added substrate far exceeds the small amount produced through the TPN⁺ or TPNH-dependent pathways, so that the differences due to the presence of the pyridine nucleotides may not become so evident as in those experiments omitting R-5-P. From the evidence shown in figure 4, although the addition of TPNH to mixtures containing R-5-P resulted in slightly greater amounts of fixation, the presence of TPN⁺, with or without methyl viologen, appeared to be somewhat inhibitory. Such inhibition is not entirely consistent with several other experiments with TPN⁺ which have shown counts equal to or slightly higher than the controls containing neither TPN⁺ nor TPNH. In any event, the inhibitory effect of TPN⁺ has only been observed in the presence of added R-5-P. The addition of TPN⁺ to reaction mixtures already containing TPNH did not cause any significant differences in the amounts of CO₂ fixed either in the presence or absence of added R-5-P. (See figure 5.) In the data presented in figure 5, the differences observed due to the presence of the pyridine nucleotide compounds are fairly obvious in the case of added R-5-P as well as in its absence. The replacement of half the TPNH by TPN⁺ did not appear to make an appreciable difference in the rates of CO₂ assimilation as compared with controls containing the usual amount of TPNH, possibly because TPNH was already in excess.

Some Factors Affecting the Carbon Dioxide Fixation System

The presence of added ribose-5-phosphate (R-5-P) precludes the necessity for the formation of ribulose-1,5-diphosphate (RuDP) the actual substrate for the carboxydismutase reaction in which CO₂ is assimilated into organic compounds (via 3-phosphoglyceraldehyde) from the complex and dilute mixture of organic components in the chloroplast extract. Such precursor compounds, or ribulose-1,5-diphosphate itself,

are present in small amounts, since some CO_2 is fixed in the absence of R-5-P and such compounds as TPN^+ or TPNH . The concentration of RuDP or its production from precursor organic molecules present in the extract is normally a rate limiting factor in the process of CO_2 fixation. Therefore, the addition of R-5-P, a compound that is rapidly converted to RuDP by spinach extracts, results in dramatic increases in rates and amounts of fixation. In the absence of added R-5-P, the extracts must depend upon the conversion of precursor molecules to RuDP prior to CO_2 fixation by the typical Calvin or reductive pentose phosphate pathway. Since the addition of either TPN^+ or TPNH to reaction mixtures without R-5-P resulted in a significant increase in CO_2 fixed, we may assume that the added compounds may be acting in the following manner: the pyridine nucleotide compounds, either TPN^+ , TPNH , or both may be required in the enzymatic conversion of organic compounds present in the extract to RuDP, and the amount of TPN^+ and/or TPNH present in the extracts is so low that the rate of this conversion is seriously reduced. Such conversion may or may not involve recycling of the products of CO_2 fixation through the necessary reductive step to form more substrate RuDP for further fixation. A number of compounds including sugars, amino acids, and nucleic acids, may contribute to the production of RuDP, and these pathways may involve the need for the pyridine nucleotide compounds.

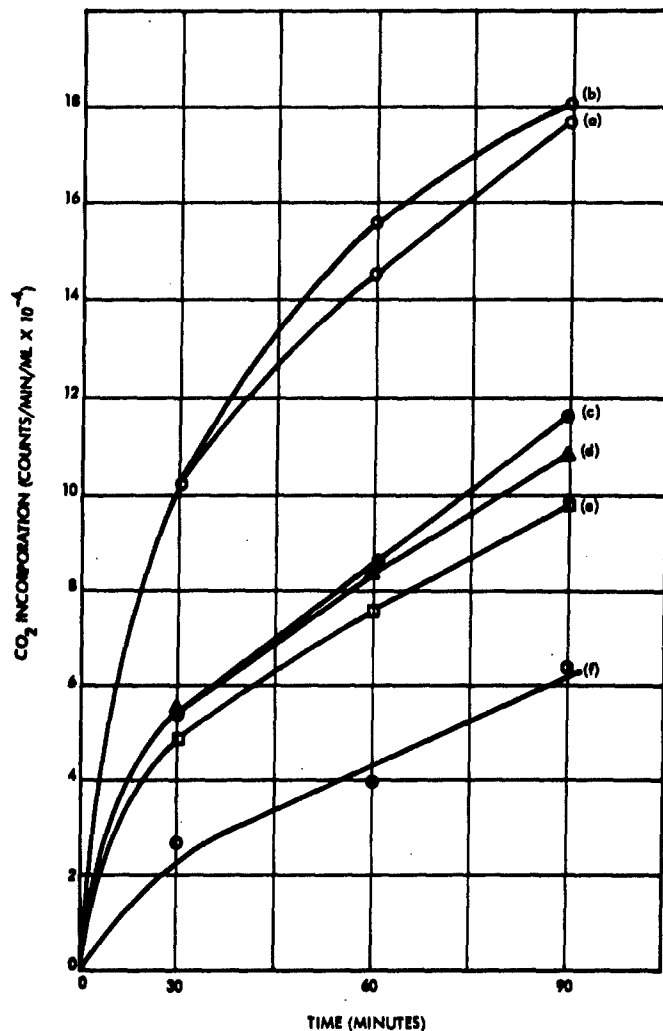


Figure 5. Rate of CO_2 Fixation in the Presence and Absence of Added Ribose-5-Phosphate. The basic reaction mixture was the same as that described under table I, except that $8 \times 10^{-4} \text{ M}$ ATP was present in all vessels. Additions include; a. $4 \times 10^{-4} \text{ M}$ TPN^+ + $4 \times 10^{-4} \text{ M}$ TPNH + $8 \times 10^{-4} \text{ M}$ R-5-P; b. $8 \times 10^{-4} \text{ M}$ TPNH + $8 \times 10^{-4} \text{ M}$ R-5-P; c. $4 \times 10^{-4} \text{ M}$ TPN^+ + $4 \times 10^{-4} \text{ M}$ TPNH ; d. $8 \times 10^{-4} \text{ M}$ R-5-P; e. $8 \times 10^{-4} \text{ M}$ TPNH ; f. No additions. Specific activity of the $[^{14}\text{C}]$ -sodium bicarbonate was $2.53 \times 10^4 \text{ cpm}/\mu\text{mole}$.

SECTION IV

CARBON DIOXIDE FIXATION AND THE ELECTROCHEMICAL REDUCTION OF PYRIDINE NUCLEOTIDES IN A COMBINED SYSTEM

INTRODUCTION

In the preceding sections, the successful electrochemical reduction of TPN^+ was demonstrated. The response of carbon dioxide fixation in modified spinach chloroplast extracts upon the addition of the ingredients which must be added in order to electrochemically generate TPNH has also been discussed. These two processes, electrochemical reduction and carbon dioxide fixation, were performed in combination to determine whether TPNH could be generated directly in the chloroplast extract, and subsequently utilized to synthesize TPNH-requiring plant substances. The concomitant generation of TPNH and carbon dioxide fixation resulted in conditions that were appropriate for the continued operation of the enzymes of the Calvin cycle. Therefore, the amounts of fixation observed in the electrolytic cell exceeded or at least equalled those of the control experiments in which TPNH was directly added to the experimental mixture.

THE COMPLETE SYSTEM

The results of preliminary experiments combining electrolysis with CO_2 fixation are tabulated in tables IV and V. Incorporation was followed by measuring the radioactivity of the nonvolatile fraction of the acidified reaction mixture.

Experiments I and II of table V were performed in the dark to exclude the possibility of photosynthetic reduction of the TPN^+ present in the electrochemical vessel by small amounts of chlorophyll still present in the extracts. These results show that the amount of CO_2 fixed in the electrolytic cell after 1 or 2 hours is significantly more than that fixed in the nonelectrolyzed TPN^+ control, and approaches or even surpasses that fixed in the TPNH control.

An analysis and comparison of the products of fixation in the three vessels as described in the two previous tables and in results to be described later indicated that the varieties of radioactively labeled chemical species formed were no different following the electrolysis than in the nonelectrolyzed control vessels containing added TPN^+ or TPNH.

Figure 6 represents a tracing of a typical chromatogram showing the relative positions of the various radioactively labeled compounds encountered in these experiments. The intensity of the individual spots varied over a wide range. For routine analysis, to be described in the sections below, $[^{14}\text{C}]$ -labeled compounds were located by autoradiography, cut out, eluted, and the radioactivity was measured in a scintillation spectrometer as described under Methods. Figures 7 and 8 represent chromatograms of deproteinized reaction mixtures sprayed with ninhydrin and ammoniacal silver nitrate solutions, respectively. Only

Table IV. Utilization of Electrochemically Generated TPNH By Chloroplast Extract. I. Sequential Electrochemical and Biochemical Operations.

Additions	Carbon Dioxide Incorporated counts per min. per ml after	
	15 min.	30 min.
1. ATP	383	704
2. ATP + TPNH	2570	4700
3. ATP + methyl viologen (ox) + TPNH	4180	8040
4. None	205	339
5. ATP + 0.5 ml elec- trolyzed reaction mixture + FTR	4760	8340

The basic reaction mixture was identical to that described in table I. The following additions were made to give a final volume of 2.5 ml: 1. 8×10^{-4} M ATP; 2. 8×10^{-4} M ATP + 8×10^{-4} M TPNH 3. 8×10^{-4} M ATP + 8×10^{-5} M mv^{++} + 8×10^{-4} M TPNH; 4. None; 5. 8×10^{-4} M ATP + 0.5 ml electrolyzed TPN⁺ solution (see Electrochemical Methods). Specific activity of [¹⁴C]-sodium bicarbonate was 4×10^4 cpm/ μ mole.

those spots showing a positive reaction with the particular spray reagent are shown, and those which contain radioactive carbon as a result of CO₂ fixation are indicated by cross hatching. Figure 9 is a representation of a similar untreated chromatogram as viewed under an ultraviolet lamp. The most prominent spots revealed under the ultra violet lamp are the adenosine phosphate compounds, AMP, ADP, and ATP, all of which are unlabeled and probably derived from the ATP added initially to the reaction mixture. Other nucleotides, nucleosides, and purine and pyrimidine bases may appear in the reaction mixtures containing more concentrated spinach extracts.

The results of a typical experiment designed to determine the relative distribution of radioactivity among the [¹⁴C]-labeled compounds in the electrolysis cell, at the end of 2 hours, are tabulated in table VI. A comparison of the electrolyzed mixture with the nonelectrolyzed TPN⁺ and TPNH controls shows some minor differences which may or may not be significant. It is important to note, however, that glucose-6-phosphate was formed in all three reaction mixtures, including the one to which TPN⁺ but not TPNH was added (TPN⁺ control). The appearance of large amounts of glyceric acid amounting to approximately half the CO₂ fixed is also worthy of note and will be recalled later. The number and variety of labeled compounds detected are relatively few.

Table V. Utilization of Electrochemically Generated TPNH By Chloroplast Extract. II. Simultaneous Electrolysis and CO₂ Fixation By Chloroplast Extracts.

Additions	Carbon Dioxide Incorporated counts per min. per ml after	
	60 min.	120 min.
I. 1. TPN ⁺ (Electrolysis)	16,630	26,630
2. TPN ⁺	12,570	19,570
3. TPNH	19,210	31,950
II. 1. TPN ⁺ (Electrolysis)	61,580	88,070
2. TPN ⁺	41,520	63,290
3. TPNH	45,520	73,130
III. 1. TPN ⁺ (Electrolysis)	33,660	
2. TPN ⁺	10,350	
3. TPNH	28,340	

The basic reaction mixture was the same as in table I. In addition, all solutions contained the following: 8×10^{-4} M ATP, 8×10^{-5} M mv^{++} , 8×10^{-4} M R-5-P, 0.5 mg/ml FTR. Further additions were as follows: 1 and 2, 8×10^{-4} M TPN⁺; 3. 8×10^{-4} M TPNH. Specific activities of the [¹⁴C]-sodium bicarbonate were: I. 6.49×10^4 , II. 1.5×10^5 , III. 1.4×10^5 cpm/ μ mole, respectively. Experiments I and II were performed in darkness. In all experiments, reaction mixture 1 was electrolyzed and contained a total of 20.0 ml, while all others were not electrolyzed and each contained 2.5 ml.

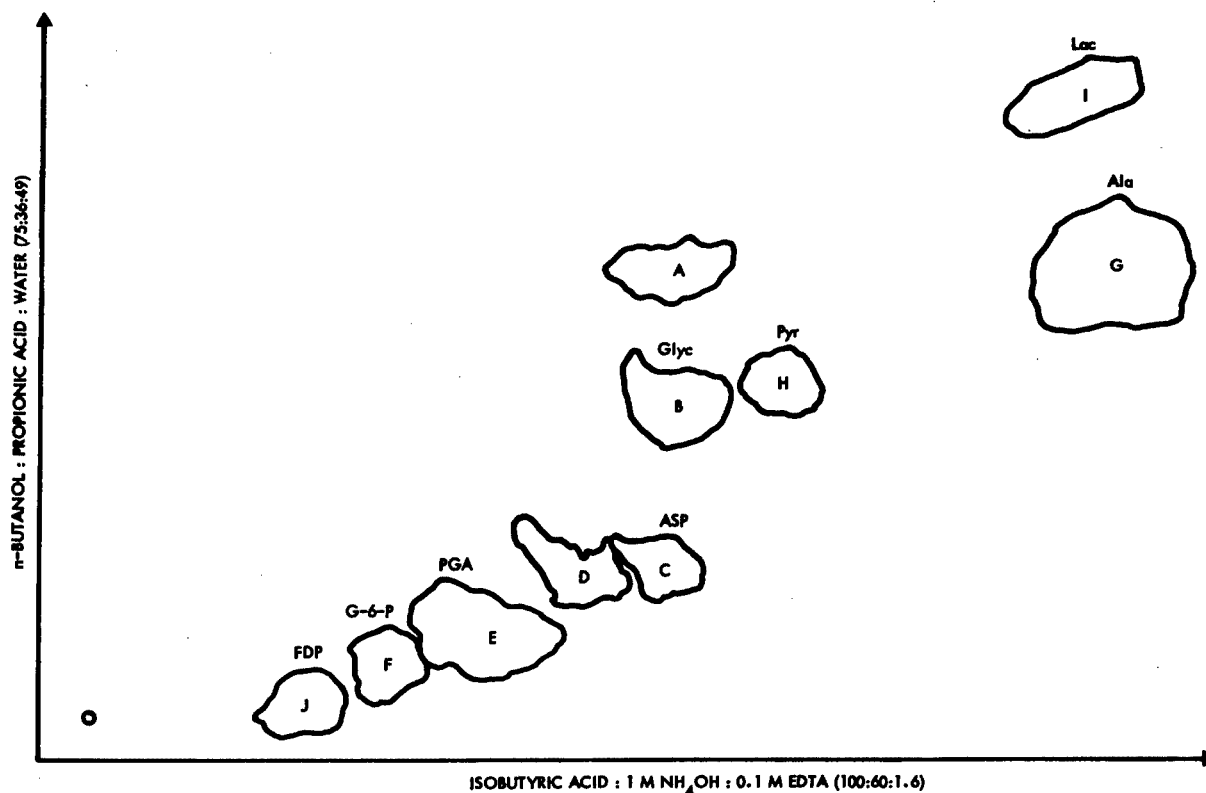


Figure 6. A Typical Distribution of Labeled Compounds Produced in Reaction Mixtures By Chloroplast Extract As Shown By Paper Chromatography. Identities of the various spots, where known, are indicated.

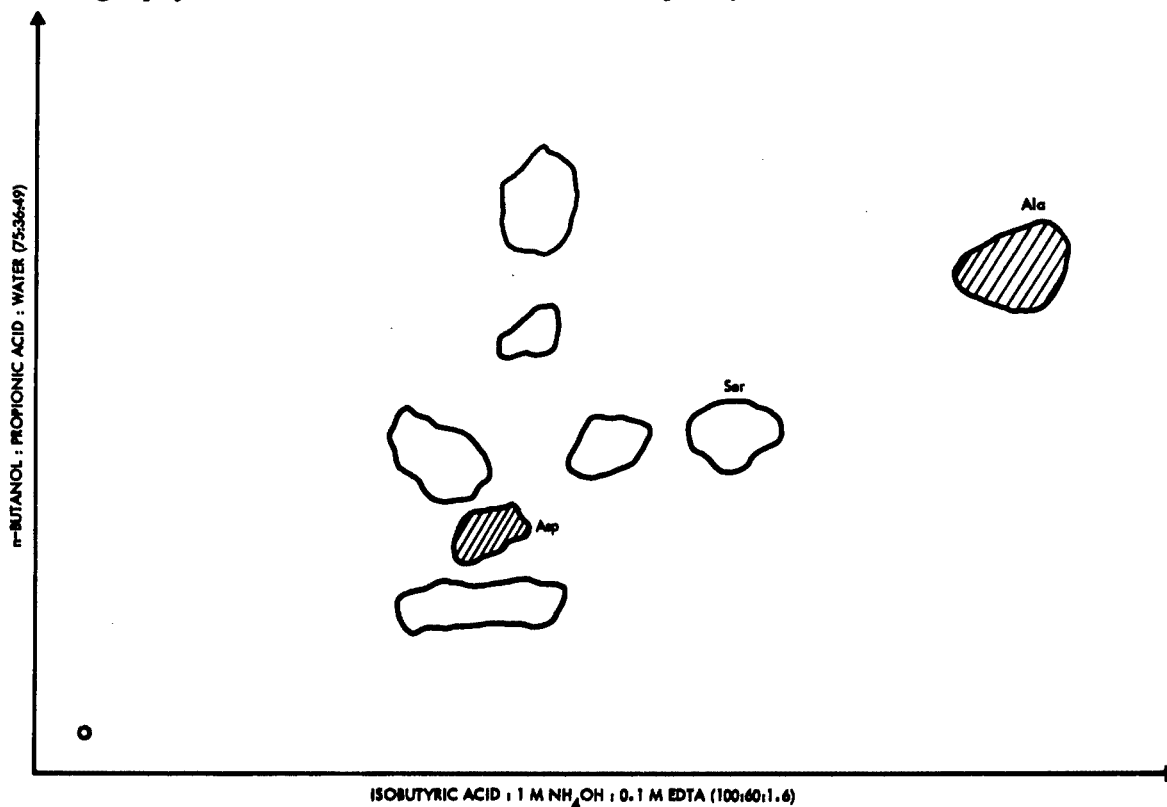


Figure 7. Chromatographic Separation of Ninhydrin-Positive Compounds Present in Deproteinized Reaction Mixtures Containing Chloroplast Extract. Radioactively labeled amino acids are indicated by cross hatching.

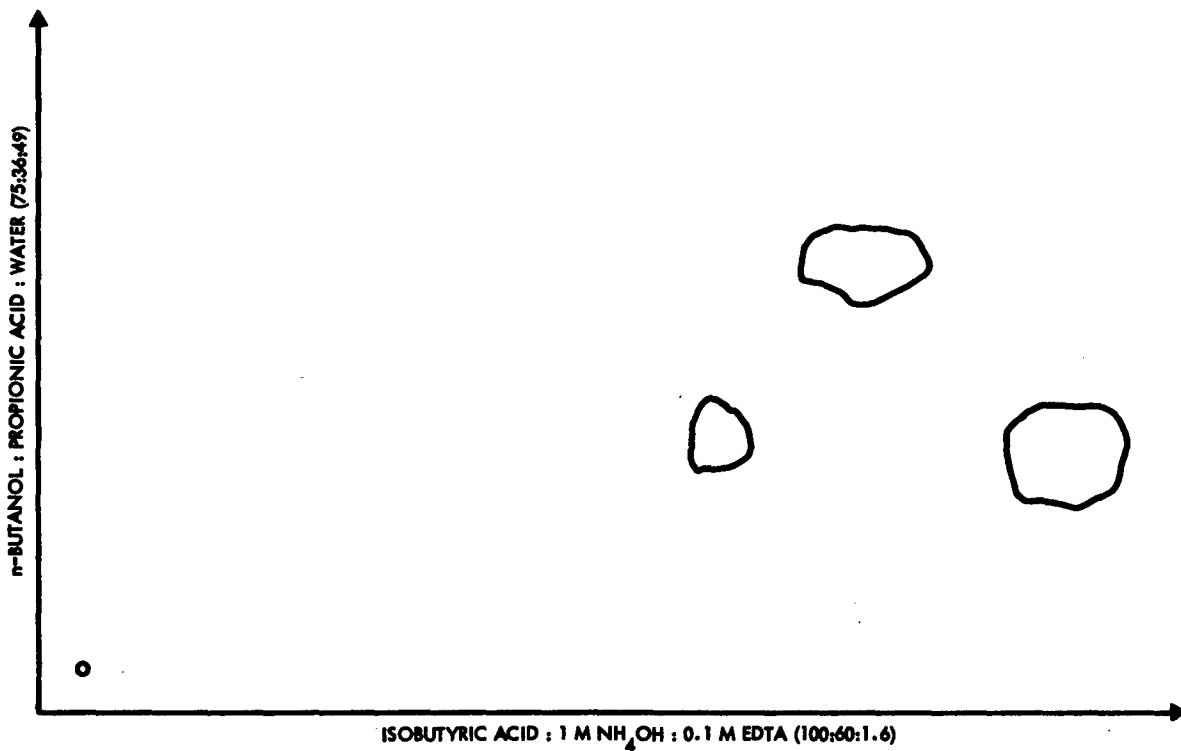


Figure 8. Chromatogram of Deproteinized Reaction Mixture Sprayed With Ammoniacal Silver Nitrate.

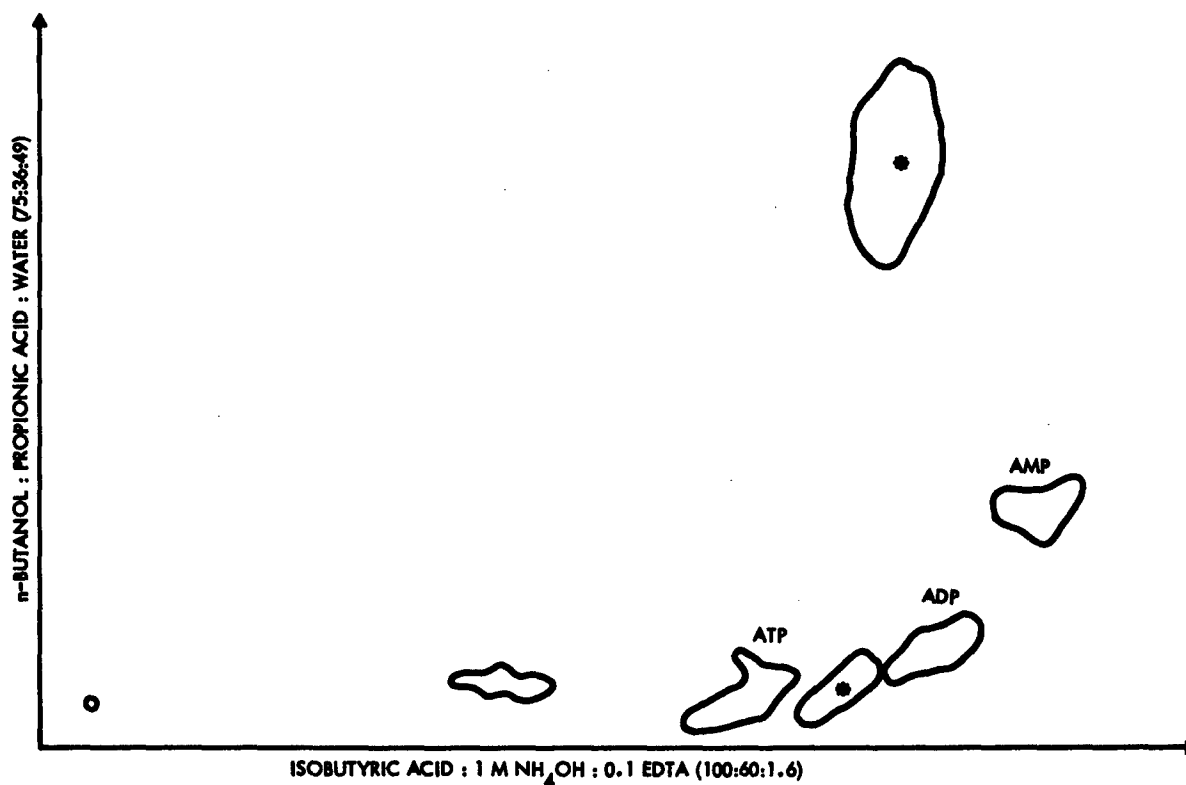


Figure 9. Chromatogram of Deproteinized Reaction Mixture As Seen Under Ultra Violet Light. Spots indicated with * were fluorescent, while all others appeared as dark spots.

THE EFFECT OF OMITTING METHYL VIOLOGEN

The effect of omitting methyl viologen in the electrolysis cell and the resulting product distribution are shown in table VII. In this particular experiment, and the one just described in table VI, the reaction mixtures were placed in the refrigerator at the end of the 2-hour experimental period and left overnight prior to deproteinization and sampling for chromatographic analysis. The result of major importance is shown in the amount of current flowing into the electrolysis cell during the 2-hour period. It amounted to only 11 percent of that observed in the complete system. This was to be expected since it was known from electrochemical studies described in an earlier section that methyl viologen was required for the electrochemical reduction of TPN^+ to TPNH.

No 3-phosphoglyceric acid nor sugar phosphates (TPNH is required for the formation of the latter) were observed on the chromatograms of these reaction mixtures. The data reported in table VI for the complete system was obtained under the same conditions, and all those reaction mixtures were found to synthesize sugar phosphates and PGA. Also note the relatively high concentrations of glyceric acid found in all reaction mixtures of table VII.

Table VI. The Complete Electrolysis System.

	Vessel Number	Integrated Current (uequiv/ml)	CO ₂ FIXED		Reaction Time (min)	DISTRIBUTION OF LABELED COMPOUNDS							
			cpm/ml	umoles/ml		PGA	Asp	Glyc	Ala	G-6-P	FDP	Unknown A	Others
I	1	2.20	8,980	0.159	120	21.2	5.6	50.2	6.9	4.4		8.3	3.4
	2		3,350	0.059	120	19.8	11.7	43.9	6.2	6.9		7.5	4.0
	3		5,340	0.095	120	9.3	33.6	42.6	2.4	2.3		6.5	3.3
II	1	1.56	111,150	0.712	120	25.4	1.0	48.8	9.4	3.8		9.3	2.3
	2		55,080	0.353	120	39.0	17.5	28.7	6.6	-		4.6	3.6
	3		61,870	0.397	120	37.1	18.6	27.1	7.8	-		4.9	4.5

The basic reaction mixture was the same as in table V. Additions were as follows: 1 and 2. $8 \times 10^{-4} \text{ M TPN}^+$; 3. $8 \times 10^{-4} \text{ M TPNH}$. Reaction mixture 1 was electrolyzed and contained a final volume of 20.0 ml in both experiments. Mixtures 2 and 3 contained 2.5 ml final volume in Experiment I and 10.0 ml in Experiment II. In Experiment I, vessels 2 and 3 were exposed to air, while in Experiment II, the vessels were flushed with argon and stoppered. Specific activity of the ^{14}C -sodium bicarbonate was 5.64×10^4 and 1.56×10^5 cpm/ μmole for experiments I and II, respectively.

Table VII. Effect of Omitting Methyl Viologen.

Vessel Number	Integrated Current (μ equiv/ml)	CO ₂ FIXED		Reaction Time (min)	DISTRIBUTION OF LABELED COMPOUNDS								
		cpm/ml	μ moles/ml		PGA	Asp	Glyc	Ala	G-6-P	FDP	Unknown A	Others*	Lactate
1	0.239	13,650	0.050	120	-	18.4	46.7	14.0			9.4	11.5	(9.7)
2		3,360	0.012	120	-	13.7	44.0	14.1			7.3	20.9	(16.2)
3		5,870	0.022	120	-	12.1	49.5	13.0			8.3	17.1	(14.9)

* Lactate figures are included in "Others".

The basic reaction mixtures were the same as those described in table V, with the exception that methyl viologen was omitted from all. Additions included: 1 and 2. 8×10^{-4} M TPN⁺; 3. 8×10^{-4} M TPNH. Solution 1 was electrolyzed. Specific activity of the [¹⁴C]-sodium bicarbonate was 2.7×10^5 cpm/ μ mole. Final volumes of the solutions were: 20.0, 2.5, and 2.5 ml for solutions 1, 2, and 3, respectively.

THE EFFECT OF OMITTING TRIPHOSPHOPYRIDINE NUCLEOTIDE

If TPN⁺ is omitted from the reaction mixture, (see table VIII) the amount of current flowing during electrolysis was approximately 55 percent of that in the complete system, but considerably more than that observed in the absence of methyl viologen. The increase in current flow if TPN⁺ is present, over that observed if TPN⁺ is omitted, is further indication that TPNH is being generated during electrolysis. the level of current flowing in the absence of added TPN⁺ suggests that there are compounds other than TPN⁺ in the extract, which may also be reduced under the conditions imposed.

Table VIII. Effect of Omitting TPN⁺.

Vessel Number	Integrated Current (μ equiv/ml)	CO ₂ FIXED		Reaction Time (min)	DISTRIBUTION OF LABELED COMPOUNDS								
		cpm/ml	μ moles/ml		PGA	Asp	Glyc	Ala	G-6-P	FDP	Unknown A	Others	
1	1.21	28,790	0.070	120	5.8	61.5	13.7	15.2	0.4		1.4	2.0	
2		21,390	0.052	120	8.8	58.4	4.6	17.5	-		0.6	10.1	
3		35,440	0.086	120	13.2	5.8	49.3	13.4	1.6		4.9	11.8	

Reaction mixtures were as described in table V, with the following additions: 1 and 2, none; 3. 8×10^{-4} M TPNH. Specific activity of [¹⁴C]-sodium bicarbonate was 1.4×10^5 cpm/ μ mole. Final volumes were: 20.0, 2.5, and 2.5 ml for solutions 1, 2, and 3, respectively. Solution 1 was electrolyzed. All solutions were held in darkness during the experiment.

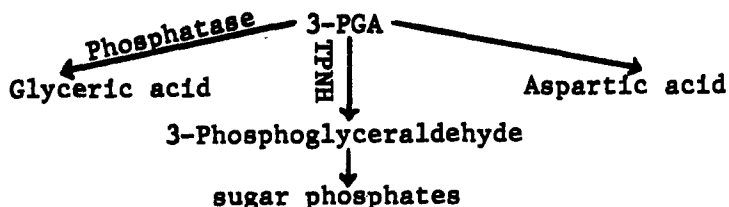
As usual, the amount of CO_2 fixed in the sample undergoing electrolysis approached that of the sample with added TPNH, while the amount fixed in the TPN^+ control was considerably lower.

In the absence of both TPN^+ and TPNH, a dramatic change was observed in the distribution of labeled compounds (vessels 1 and 2 in table VIII). The glyceric acid spot diminished in relative intensity while the aspartic acid spot became the most intensely labeled spot on the chromatogram. In the TPNH control (vessel 3 in table VIII), however, the distribution was similar to that seen in previously described experiments. In the presence of the pyridine nucleotide compounds (TPN^+ or TPNH), there was a tendency for glyceric acid to accumulate in the extracts, while in the absence of these compounds, aspartic acid was consistently found to accumulate. This tendency is noteworthy, because the currently accepted biochemical pathway for the formation of glyceric acid does not involve a reductive step. Aspartic acid may be formed in either of two ways, one of which requires reduced pyridine nucleotides. If this is so, then the data suggests that inhibitory or activation mechanisms are operative. The formation of aspartic acid from 3-PGA has been shown to involve the fixation of another mole of CO_2 according to the following proposed pathway:



The incorporation of the extra mole of CO_2 for every mole of aspartic acid formed by this pathway would result in deceptively high total counts in those reaction mixtures in which aspartic acid was present in large amounts.

Among several possible explanations for the shift of radioactivity from glyceric to aspartic acids is the interesting one: TPN^+ and TPNH interfere with the aspartic acid pathway, resulting in accumulation of 3-phosphoglyceric acid. As the latter compound accumulates, it is enzymatically hydrolyzed by indigenous phosphatases. If TPN^+ and TPNH are not present, synthesis of aspartic acid occurs rapidly enough to remove the 3-PGA before it can be hydrolyzed by the competing phosphatases. Alternatively, TPN^+ and TPNH could contribute to the activation of the phosphatases.



At the same time, regardless of possible regulatory functions of the pyridine nucleotides in determining relative activities of these side pathways, TPNH is absolutely required in the process of reduction to form sugar phosphates and recycling of these compounds to allow further fixation of CO₂.

THE EFFECT OF OMITTING METHYL VIOLOGEN AND TRIPHOSPHOPYRIDINE NUCLEOTIDE

If both methyl viologen and TPN⁺ are omitted from the complete reaction mixture, the results are as shown in table IX. The very low current flow was, as explained earlier, due to the absence of methyl viologen. Once again, in those reaction mixtures which contained neither TPN⁺ nor TPNH, the aspartic acid spots were heavily labeled with radioactive carbon while only small amounts of labeled glyceric acid were found. On the other hand, if either the oxidized (TPN⁺) or reduced (TPNH) pyridine nucleotide was present, the situation was reversed: large amounts of labeled glyceric acid appeared with only small amounts of labeled aspartic acid. Labeled sugar phosphate was found in the electrolyzed samples and in all the controls containing either TPN⁺ or TPNH. In that reaction mixture (2), which was identical in all respects to the electrolytic cell except for electrolysis, there was no evidence for labeled glucose-6-phosphate. We assume that under electrolysis conditions, sufficient TPN⁺ was present in the natural extract to synthesize a small amount of sugar phosphate. Enzyme stability under reducing conditions may also be involved.

Table IX. Effect of Omitting Both Methyl Viologen and TPN⁺.

Vessel Number	Integrated Current (μequiv/ml)	CO ₂ FIXED		Reaction Time (min)	DISTRIBUTION OF LABELED COMPOUNDS							
		cpm/ml	μmoles/ml		PGA	Asp	Glyc	Ala	G-6-P	FDP	Unknown A	Others
1	0.168	45,730	0.293	120	22.8	65.2	4.0	4.8	0.2		-	3.0
2		35,300	0.226	120	23.3	63.7	3.7	6.0	-		-	3.3
3		47,180	0.302	120	24.8	4.2	46.4	8.3	6.8		6.2	3.3
4		37,710	0.242	120	30.1	3.9	38.8	10.5	8.4		5.7	2.6
5		43,680	0.280	120	26.9	2.2	47.0	8.4	6.0		7.0	2.5

The basic reaction mixture was the same as that described in table V, with the following additions or deletions: methyl viologen was omitted from all vessels, and FTR was omitted from vessels 4 and 5. Further additions included: 1 and 2. None; 3 and 5. 8×10^{-4} M TPNH; 4. 8×10^{-4} M TPN⁺. All vessels were held in darkness during the experiment. Specific activity of the [¹⁴C]-sodium bicarbonate was 1.56×10^5 cpm/μmole. Solution 1 was electrolyzed. Final volumes were 20.0 ml for vessel 1 and 2.5 ml for solutions 2-5.

EFFECT OF PURGING THE REACTION VESSEL WITH ARGON

In most of the preliminary experiments, only the electrolytic cells were continuously purged with argon to provide an inert atmosphere. This was necessary to avoid reoxidation of the reduced methyl viologen produced at the electrode surface. Control experiments were done in separate small Erlenmeyer flasks or test tubes without purging with argon. The possibility that the purging process had some beneficial effect on the enzymes involved in CO_2 fixation was strengthened by the results shown by a comparison between the purged and nonpurged vessels 3 and 4 of table X. Although there appeared to be no major differences in the distribution of labeled compounds, the total counts were observed to be somewhat higher in the purged control. It may be speculated that the absence of oxygen in the vessel prolonged the activity of one or many of the enzymes concerned with CO_2 fixation, which require that certain critical chemical groups (i.e., sulfhydryl groups) remain in the reduced state.

Table X. Effect of Purging Control Samples With Argon.

Vessel Number	Integrated Current ($\mu\text{equiv/ml}$)	CO_2 FIXED		Reaction Time (min)	DISTRIBUTION OF LABELED COMPOUNDS							
		cpm/ml	$\mu\text{moles/ml}$		PGA	Asp	Glyc	Ala	G-6-P	FDP	Unknown A	Others
1	1.83	57,020	0.366	120	29.7	7.1	36.9	5.7	6.1	1.6	7.0	5.9
2		58,860	0.377	120	20.4	5.2	46.3	11.1	4.3		9.1	3.6
3		65,430	0.419	120	13.4	9.1	54.2	7.7	2.5		11.1	2.0
4		43,640	0.280	120	19.4	6.1	46.7	9.0	4.1		9.1	5.6

Reaction mixtures were the same as in table V, with the following additions: 1 and 2. $8 \times 10^{-4} \text{ M TPN}^+$; 3 and 4. $8 \times 10^{-4} \text{ M TPNH}$. All mixtures except 4 were continuously purged with argon during the experiment. Mixture 4 was exposed to air. The reaction was performed in darkness. Final volumes of the various reaction mixtures were: 20.0 ml for vessels 1 (electrolyzed), 2, and 3, and 2.5 ml in vessel 4. Specific activity of the $[^{14}\text{C}]$ -sodium bicarbonate was $1.56 \times 10^5 \text{ cpm}/\mu\text{mole}$.

EFFECT OF OMITTING EXOGENOUS BIOCHEMICAL REDUCTANTS

The striking shift in the relative concentrations of aspartic acid and glyceric acid observed if TPN^+ and TPNH are omitted from the reaction mixture, though an interesting phenomenon, is not obviously related to the reducing properties of TPNH because (a) the steps in the formation of glycerate do not involve reduction and (b) if a pyridine nucleotide reduction is required for aspartic acid formation the observed effect of TPNH is in the wrong direction. Furthermore, the appearance of labeled sugar phosphates, known to require reductive processes for their formation from labeled 3-PGA, appear if either TPN^+ or TPNH are present. These results suggest that although the pyridine nucleotide

compounds were present in the extracts only in limiting concentrations, there was a ready source of compounds either in the extract or added during the routine procedure that could contribute to the reduction of exogenous TPN⁺.

The reaction mixture chosen for the demonstration of CO₂ fixation was essentially that used by Arnon and coworkers in their earlier studies of the same phenomenon. Their procedure called for the routine addition of cysteine, ascorbic acid, and glucose-1-phosphate, attempting to maintain optimal conditions for the operation of the enzymes responsible for fixation of CO₂. More recently, (Kalberer, Buchanan, and Arnon, 1967) several of the compounds of the basic reaction mixture, including those listed above, were found to be nonessential for these enzymatic reactions. In our own attempt to eliminate all possible sources of reduction except for the electrode surface, or deliberately added TPNH, the effects of these compounds were among the first to be seriously considered. The omission of these chemicals, all of which could conceivably cause the reduction of added TPN⁺, resulted in the data shown in table XI. The final sample, as reported in the table, was taken at the end of 4 hours. Only a very small amount of labeled glucose-6-phosphate was found in the reaction mixture containing no added TPN⁺ or TPNH (4), while all others, receiving either TPN⁺ or TPNH, showed the usual amounts of labeled G-6-P. Thus, the mere omission of the reductants listed above did not solve the problem, and there seem to be other reductants besides these compounds in the system.

Table XI. Effect of Omitting Ascorbate, Cysteine, and Glucose-1-Phosphate.

Vessel Number	Integrated Current (μequiv/ml)	CO ₂ FIXED		Reaction Time (min)	DISTRIBUTION OF LABELED COMPOUNDS								
		cpm/ml	μmoles/ml		PGA	Asp	Glyc	Ala	G-6-P	FDP	Unknown A	Others	
I	1	2.78	74,880	0.480	240	38.9	-	33.6	13.6	4.4	-	6.8	2.7
	2		46,890	0.300	240	32.5	1.1	30.3	17.6	6.9	2.6	5.7	3.3
	3		63,880	0.409	240	31.9	1.0	29.0	21.7	4.9	1.9	5.6	4.0
	4		41,270	0.264	240	25.4	32.2	6.6	28.0	1.6	-	1.1	5.1
II	1	2.82	91,810	0.588	240	18.6	1.0	48.0	16.2	2.7	1.2	10.1	2.2
	2		68,440	0.439	240	13.5	1.3	42.9	22.0	2.4	3.1	9.1	5.7
	3		81,450	0.522	240	12.6	2.3	47.2	20.5	1.9	-	10.6	4.9
	4		51,910	0.333	240		most intense spot			Not Done			

The basic reaction mixture was the same as that described in table V, with the exception that G-1-P, cysteine, and ascorbic acid were omitted. The following additions were made: 1 and 2. 8×10^{-4} M TPN⁺; 3. 8×10^{-4} M TPNH; 4. None. All solutions were continuously purged with argon during the reaction. Final volumes were: For experiment I: vessel 1. 20.0 ml, and vessels 2-4. 5.0 ml. Experiment II; 22.4, 11.2, 11.2, and 11.6 ml for vessels 1-4, respectively. Specific activity of the [¹⁴C]-sodium bicarbonate was 1.56×10^5 cpm/μmole.

OMISSION OF REDUCTANTS IN A SYSTEM WITHOUT RIBOSE-5-PHOSPHATE

Experiments similar to those just described were also conducted in the absence of ribose-5-phosphate to ascertain whether this compound could be acting not only as a source for ribulose-1,5-diphosphate required for CO₂ fixation, but also for a source of electrons for the reduction of TPN⁺. The results of these experiments are shown in table XII. Samples were removed for chromatographic analysis at the 4-hour mark, and because of the low levels of fixation, required extended times for radioautography (3 weeks). No labeled sugar phosphates were detected in any of the reaction mixtures, and no significant differences were observed between the TPN⁺ and TPNH controls. The labeled 3-PGA found in the electrolyzed mixtures is increased compared with the controls. In the first experiment, the increase of 3-PGA probably arose as the result of decreased dephosphorylation to form glyceric acid under the conditions during electrolysis. The results of the second experiment, however, failed to indicate decreased amounts of glyceric acid in the electrolyzed reaction mixture.

Table XII. Omission of Ribose-5-Phosphate From Complete System.

Vessel Number	Integrated Current (μequiv/ml)	CO ₂ FIXED cpm/ml	μmoles/ml	Reaction Time (min)	PGA	Asp	Glyc	Ala	G-6-P	FDP	Unknown A	Others
I												
1	3.00	19,690	0.126	240	46.2	1.0	3.0	27.9	--	--	16.6	5.3
2		13,040	0.084	240	7.1	3.4	38.6	33.4	--	--	7.0	10.5
3		16,660	0.107	240	5.7	1.9	33.4	39.9	--	--	6.9	12.2
4		4,120	0.026	240	--	69.6	5.4	25.0	--	--	--	--
II												
1	3.20	12,850	0.082	240	11.7	23.6	35.0	17.7	--	--	6.3	5.7
2		8,900	0.057	240	--	26.5	37.7	18.4	--	--	7.7	9.7
3		10,390	0.067	240	--	29.3	31.2	20.6	--	--	6.6	12.3
4		6,730	0.043	240	--	38.6	33.9	14.0	--	--	7.0	6.5

The basic reaction mixture was the same as in table XI, except that R-5-P was omitted from all vessels. Additions included: Experiment I. 1 and 2. 8×10^{-4} M TPN⁺; 3. 8×10^{-4} M TPNH; 4. None. Experiment II. 1 and 4. 8×10^{-4} M TPN⁺; 2. 8×10^{-4} M TPN⁺ + 4×10^{-4} M TPNH. 3. 8×10^{-4} M TPNH. Specific activity of [¹⁴C]-sodium bicarbonate was 1.56×10^5 cpm/μmole. All solutions were continuously purged during the reaction. Final volumes in both experiments I and II were as follows: vessel 1. 20.0 ml; vessels 2-4. 5.0 ml.

KINETIC CHARACTERISTICS OF THE CARBON DIOXIDE FIXATION SYSTEM DURING ELECTROLYSIS

Data from studies of the relative rates of fixation of CO_2 in the electrolysis cell and the control vessels are shown in figures 10-12. Figure 10 depicts an operating system without the exogenous biochemical reductants described in a previous section, but with added ribose-5-phosphate. Figure 11 and 12, on the other hand, show the results obtained in a similar system lacking the ribose-5-phosphate. Initial rates of CO_2 fixation were considerably slower in the electrolysis cell than in the controls which were not electrolyzed. But, by the end of the 2-hour period, the amount fixed in the electrolysis cell was second only to the TPNH control, and at the 4-hour period, was the highest of all. Extension of the experimental period and continuing electrolysis for up to 8 hours (figure 12) resulted in no significant increase in the amount of CO_2 fixed over those amounts found after 4 hours in all vessels.

The greatest amount of fixation was consistently found in the electrolysis cell originally containing TPN^+ . Although initial rates of fixation were slower in the electrolysis vessel than in the controls, the CO_2 fixing ability of the extracts appeared to be sustained over longer periods of time so that the total amount of fixation was greater.

Taking the amount of CO_2 fixed in the electrolytic vessel as a base figure, the relative amounts of CO_2 fixed in the various controls of an operating system such as those described above are shown in table XIII. Despite the fact that the absolute amounts of CO_2 fixed are nearly four times as great in the presence of R-5-P, the same relative amounts of fixation are observed in the TPN^+ and TPNH controls. The major difference between the relative amounts fixed in the two sets of conditions was observed in the control containing neither TPN^+ nor TPNH. There was a marked increase in the relative amount of CO_2 fixed in the "blank" of the operating system employing R-5-P.

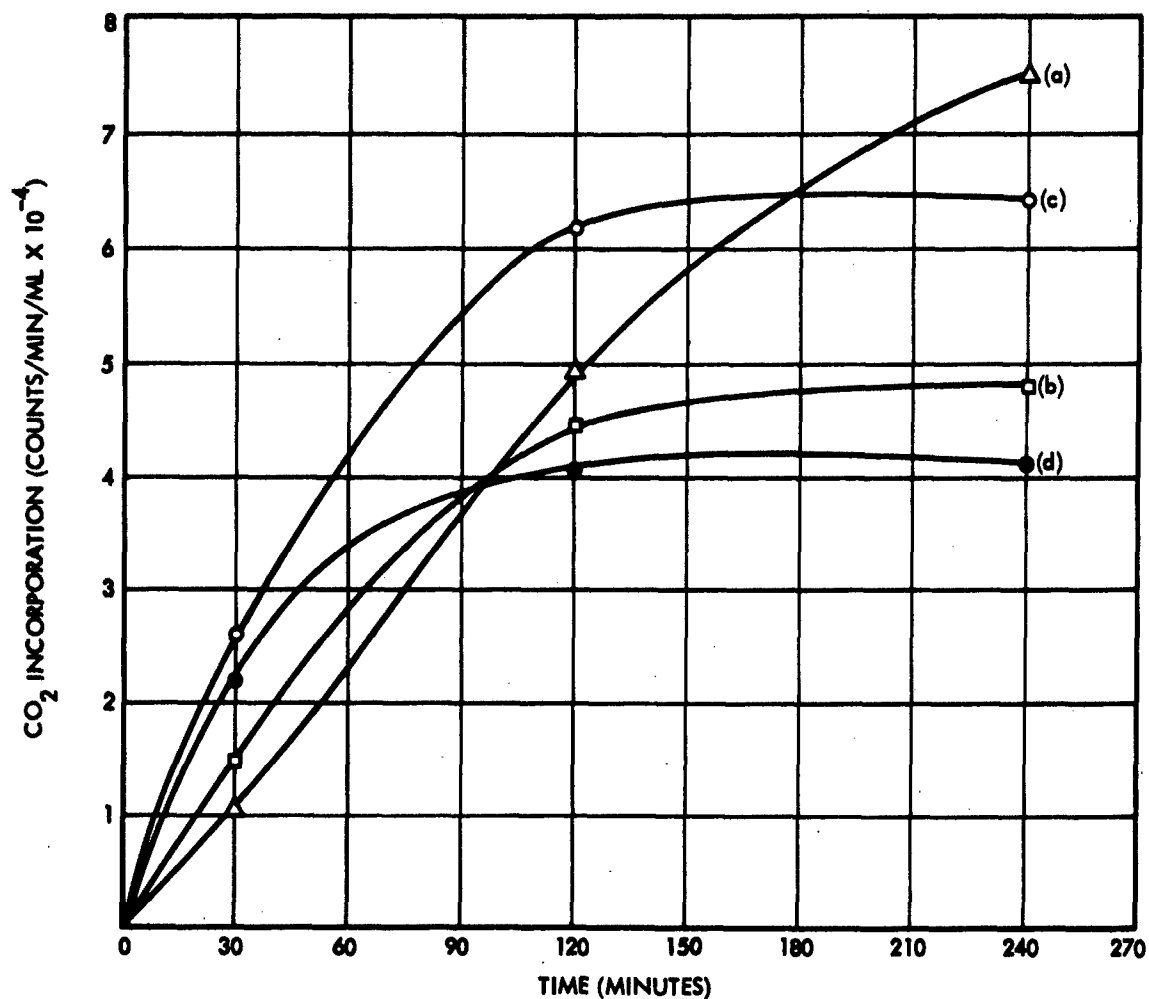


Figure 10. Rate of CO_2 Fixation in Complete System with Ribose-5-Phosphate. Conditions were the same as those described under table XI, with the following additions: a. $8 \times 10^{-4} \text{ M TPN}^+$ (with electrolysis); b. $8 \times 10^{-4} \text{ M TPN}^+$; c. $8 \times 10^{-4} \text{ M TPNH}$; d. no additions. Specific activity of the $[^{14}\text{C}]$ -sodium bicarbonate was $1.56 \times 10^5 \text{ cpm}/\mu\text{mole}$.

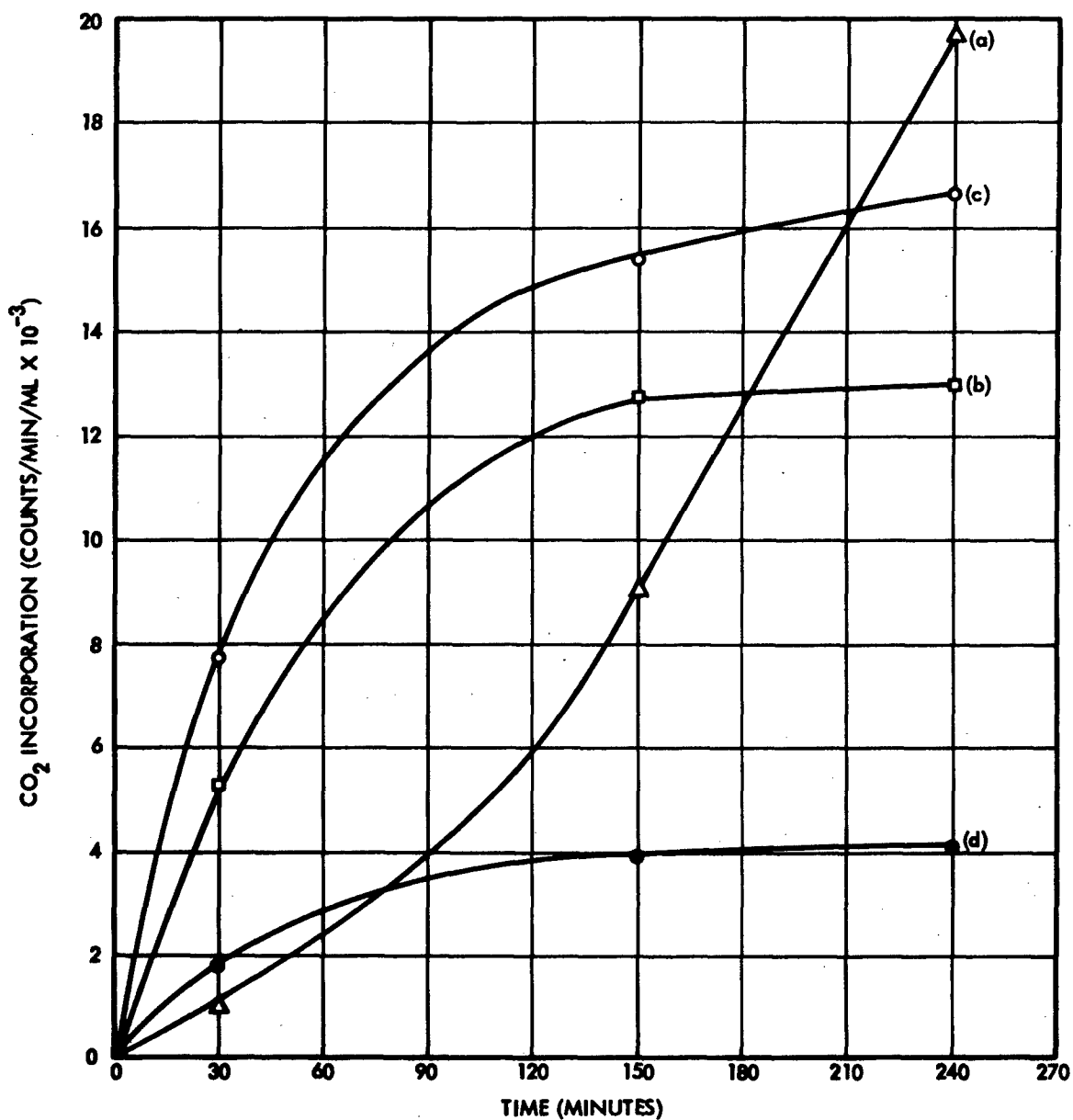


Figure 11. Rate of CO_2 Fixation in System Without Ribose-5-Phosphate. Conditions were those described under table XII. a. $8 \times 10^{-4} \text{ M TPN}^+$ (with electrolysis); b. $8 \times 10^{-4} \text{ M TPN}^+$; c. $8 \times 10^{-4} \text{ M TPNH}$; d. no additions. Specific activity of the $[^{14}\text{C}]$ -sodium bicarbonate was $1.56 \times 10^5 \text{ cpm}/\mu\text{mole}$.

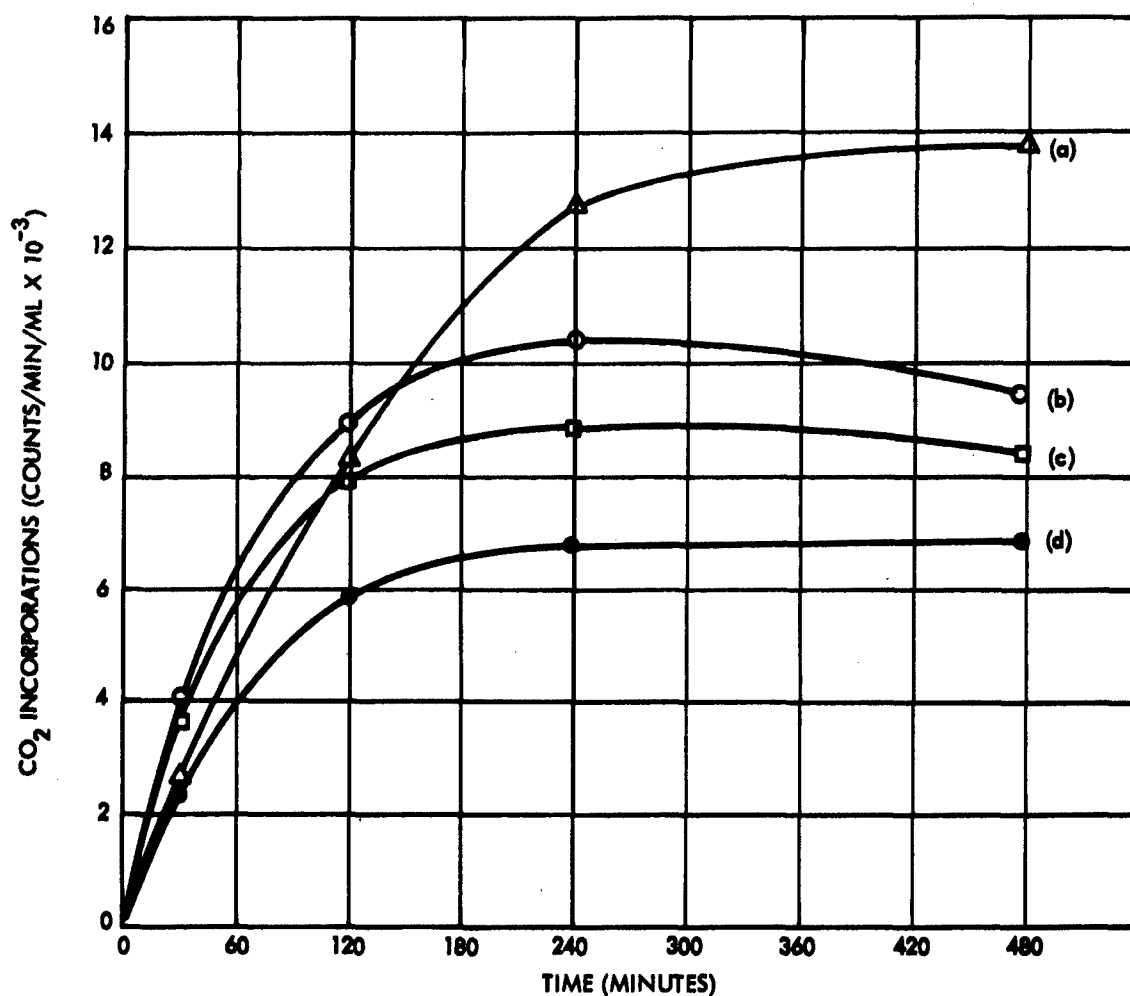


Figure 12. Extended Rate Study of CO_2 Fixation in System Without Ribose-5-Phosphate. Conditions were those described under table XII.
 a. 8×10^{-4} M TPN⁺ (with electrolysis); b. 8×10^{-4} M TPNH;
 c. 4×10^{-4} M TPNH + 8×10^{-4} M TPN⁺; d. 8×10^{-4} M TPN⁺.
 Specific activity of the ^{14}C -sodium bicarbonate was 1.56×10^5 cpm/ μmole .

Table XIII. Comparison of Systems With and Without Ribose-5-Phosphate.

Additions	with R-5-P		without R-5-P	
	cpm/ml	relative % of high value	cpm/ml	relative % of high value
1. TPN ⁺ + electrolysis	74,880	100	19,690	100
2. TPN ⁺	46,890	65	13,040	66
3. TPNH	63,880	89	16,660	85
4. No addition	41,270	57	4,120	21

Experimental conditions and data were those described in figures 7 and 8. Specific activity of [¹⁴C]-sodium bicarbonate was 1.56×10^5 cpm/ μ mole.

SECTION V

CONCLUSIONS

We have demonstrated that electrochemical energy can substitute for photochemical energy in the reduction of TPN^+ to TPNH within cell-free chloroplast extracts. Furthermore, the TPNH produced stimulated TPNH-dependent incorporation of carbon dioxide into plant substances.

TPN^+ can be electrochemically reduced to produce enzymatically-active TPNH through mediation of methyl viologen and ferredoxin-TPN-reductase.

Methyl viologen is an essential part of the electrochemical process by which TPNH is formed from TPN^+ . It does not inhibit the process of carbon dioxide fixation by spinach chloroplast extracts. On the contrary, in the presence of TPNH, methyl viologen has been shown to accelerate the assimilation of carbon dioxide.

Electrolysis, conducted concurrently with carbon dioxide fixation, did not alter the types of labeled compounds normally produced as a result of the fixation of radioactively-labeled carbon dioxide by chloroplast extracts. The relative amounts of the various products and the total amount of carbon dioxide fixed in the electrolysis cell resembled the results obtained with authentic TPNH.

Initial rates of carbon dioxide fixation were slower in the electrolysis cell than in the nonelectrolyzed reaction mixtures, but the activity of the extracts was apparently prolonged under the conditions imposed during electrolysis. The final amount of carbon dioxide fixed after 4 hours in the electrolyzed reaction mixture exceeded that in nonelectrolyzed mixtures containing either TPN^+ or TPNH.

The principal products of carbon dioxide fixation by chloroplast extracts are: 3-phosphoglyceric acid, glucose-6-phosphate, fructose-1,6-diphosphate, aspartic acid, glyceric acid, alanine, pyruvic acid, and lactic acid. As much as 90% of the final product may appear as 3-phosphoglyceric acid and either aspartic acid or glyceric acid. The relative amounts of aspartic acid and glyceric acid show a marked variation, dependent on the addition of exogenous TPN^+ or TPNH. The addition of either or both of these pyridine nucleotides to the reaction mixture caused a significant decrease in the amount of aspartic acid and a corresponding increase in the amount of glyceric acid produced.

The appearance of labeled fructose-1,6-diphosphate and glucose-6-phosphate among the products of carbon dioxide fixation does not show a distinct dependence on the addition of TPNH or the production of electrochemically-generated TPNH. The sugar phosphates may be found if either TPN^+ or TPNH is present. The regular appearance of such labeled sugar phosphates in nonelectrolyzed reaction mixtures containing TPN^+ suggests that TPN^+ can be reduced chemically or enzymatically

by some component(s) present in the reaction mixture. Results indicate that the source of this reducing substance is a natural component of the extract itself.

Until steps are undertaken to remove the endogenous reductants present in the chloroplast extracts, the appearance of sugar phosphates among the products of carbon dioxide fixation cannot be used as a criterion to distinguish between the presence of TPN^+ or TPNH .

SECTION VI

RECOMMENDATIONS FOR FUTURE STUDY

As a continuation of the studies reported in the preceding sections, we recommend consideration of the following:

Continued study of the system of concurrent electrolysis and carbon dioxide incorporation to better define the conditions under which the formation of desirable compounds is favored, and most efficient utilization of energy is attained.

Study methods for generating ATP within the electrolysis cell, thus providing a system that can entirely substitute for the photochemical process in nature.

Determine the reasons for the increased amounts of carbon dioxide assimilated in the electrolysis cell as compared to the nonelectrolyzed reaction mixtures containing TPNH.

Attempt to improve the yield of plant products formed by the fixation of carbon dioxide. Studies should be undertaken to find conditions, which would allow more effective functioning of the enzymes of the Calvin cycle, so as to regenerate ribulose-1,5-diphosphate (the immediate substrate for carbon dioxide fixation) from the products of fixation.

Attempt to influence the course of carbon dioxide fixation so as to favor the formation of relatively large amounts of desirable food products. This may best be accomplished by working with purified or semi-purified enzyme preparations, removing or inactivating the enzymes controlling undesired side reactions.

Determine the reason for the stimulation of carbon dioxide fixation by methyl viologen in the presence of TPNH.

Study the effect of the pyridine nucleotide compounds on the enzymes responsible for the formation of aspartic acid and glyceric acid, to determine why the presence of these pyridine nucleotides causes a decrease in aspartic acid and accumulation of large amounts of glyceric acid.

Attempt to increase the stability of the enzymes required for cyclic carbon dioxide fixation by modifying existing enzymes and by independent synthesis.

APPENDIX

SUMMARY OF DIFFERENT VIEWS ON PHOTOSYNTHETIC PATHWAYS IN GREEN PLANTS

The scheme presented by Clayton (1963, 1965) is shown in figure 1. As a result of excitation of chlorophyll by light, a primary oxidant (ferredoxin has been suggested) is reduced by a primary reductant (e.g., water or cytochrome *f*). In the course of the oxidation-reduction reactions, ATP is synthesized. This, together with reduction products such as NADH, DPNH, and ferredoxin, is used in synthetic activities.

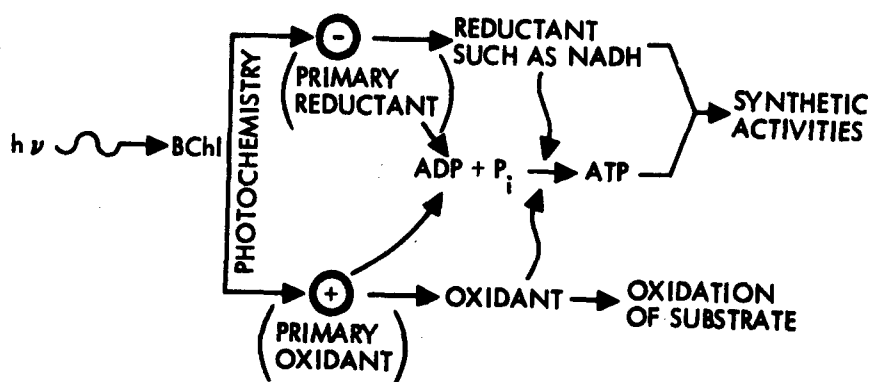


Figure 1. Bacterial photosynthesis - a modification of van Neil's original formulation (Clayton, Science 149, 1965, p. 1347).

A scheme accentuating the distinction between light requiring reactions and reactions which do not require light is shown in figure 2 (Arnon, 1965). Only the initial reduction steps require light.

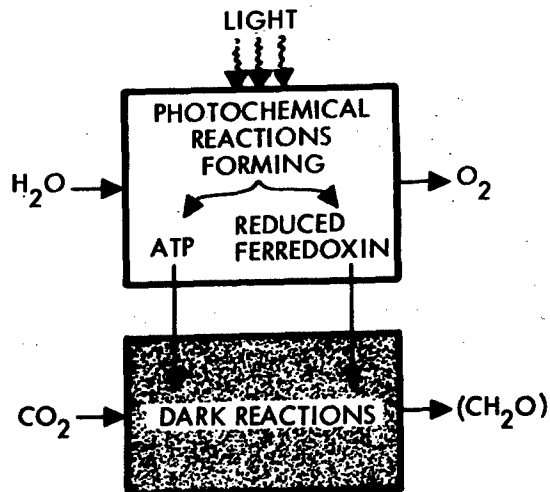


Figure 2. Diagrammatic representation of the light and dark reactions of photosynthesis in chloroplasts (Arnon, Science, 149, 1965, p. 1468)

Involvement of chlorophyll with two different excitation energy requirements is emphasized by Clayton (1963, 1965), figure 3:

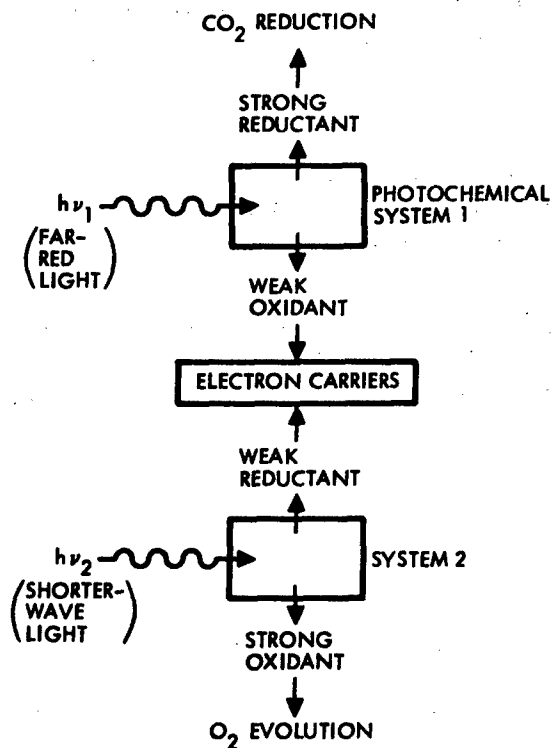


Figure 3. Outline of a series formulation for green plant photosynthesis (Clayton, Science 149, 1965, p. 1348)

Photoreduction can be bypassed by reduction with hydrogen, either by dark adaptation of algae, or by using an externally supplied hydrogenase (Arnon, 1959), figure 4:

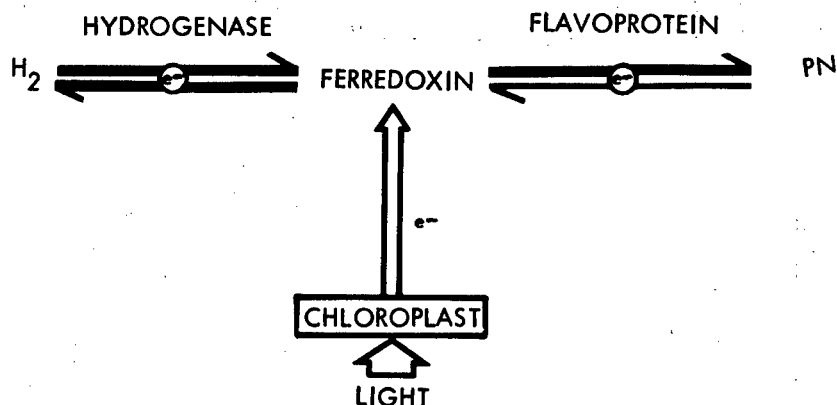


Figure 4. Diagrammatic representation of the role of ferredoxin in photochemical reduction of pyridine nucleotides (PN) and in the biological production and consumption of H₂ (Arnon, Science 149, 1965, p. 1466)

The Arnon formulation for noncyclic and cyclic phosphorylation (Arnon, Tsujimoto, McSwain, 1965), figures 5 and 6:

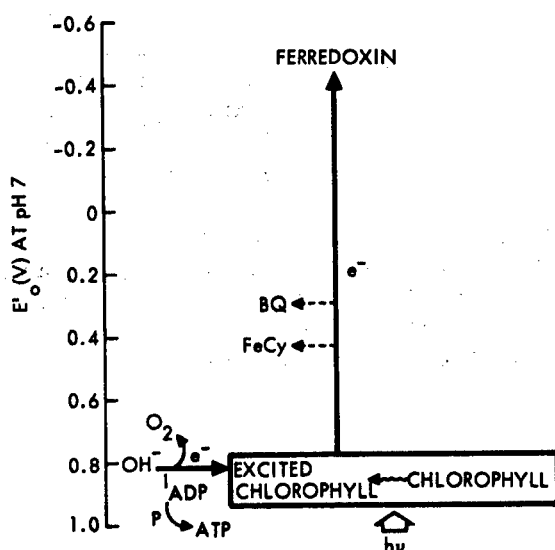


Figure 5. Scheme for non-cyclic photophosphorylation of the plant type (Arnon, et al., Nature 207, 1965, p. 1371)

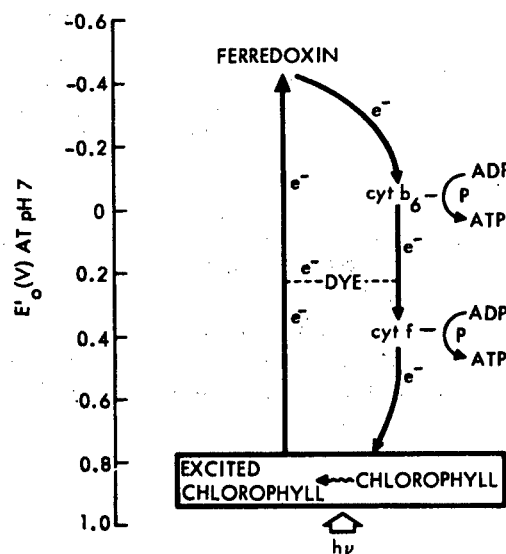


Figure 6. Scheme for cyclic photophosphorylation (Arnon, et al., Nature 207, 1965, p. 1369)

The scheme for combined cyclic and noncyclic phosphorylation systems (Arnon, Tsujimoto, and McSwain, 1965) is shown in figure 7. How the cyclic system can act as a noncyclic system in the presence of an added reduced dye (Arnon, Tsujimoto, and McSwain, 1965) is shown in figure 8.

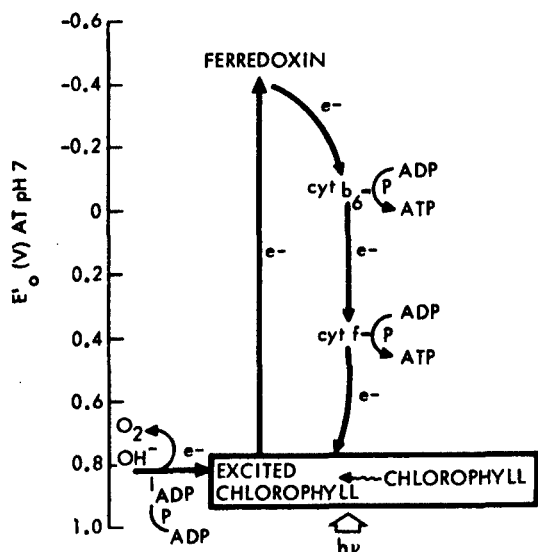


Figure 7. Joint scheme for cyclic and noncyclic photophosphorylation in chloroplasts (Arnon, *et al.*, *Nature* 207, 1965, p. 1372)

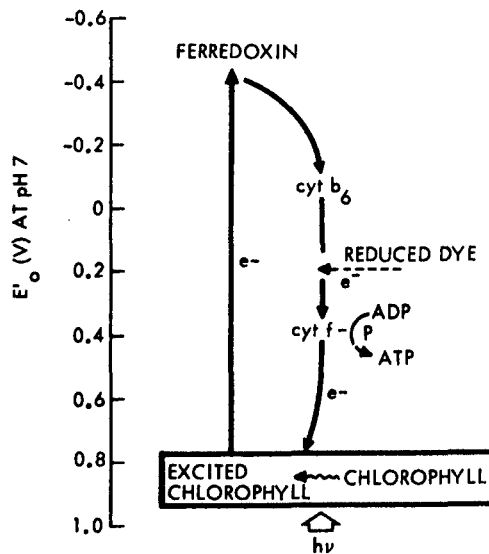


Figure 8. Scheme for noncyclic photophosphorylation of the bacterial type (Arnon, *et al.*, *Nature* 207, 1965, p. 1370)

Vernon and Avron (1965), figure 9, have formulated photoreduction and photosynthesis in the following manner. The loop including pigment system 1 looks like Arnon's cyclic phosphorylation system, and pigment system 2 like Arnon's noncyclic phosphorylation scheme, except that this is a series system and Arnon's combined scheme (figure 7) looks like a parallel formulation.

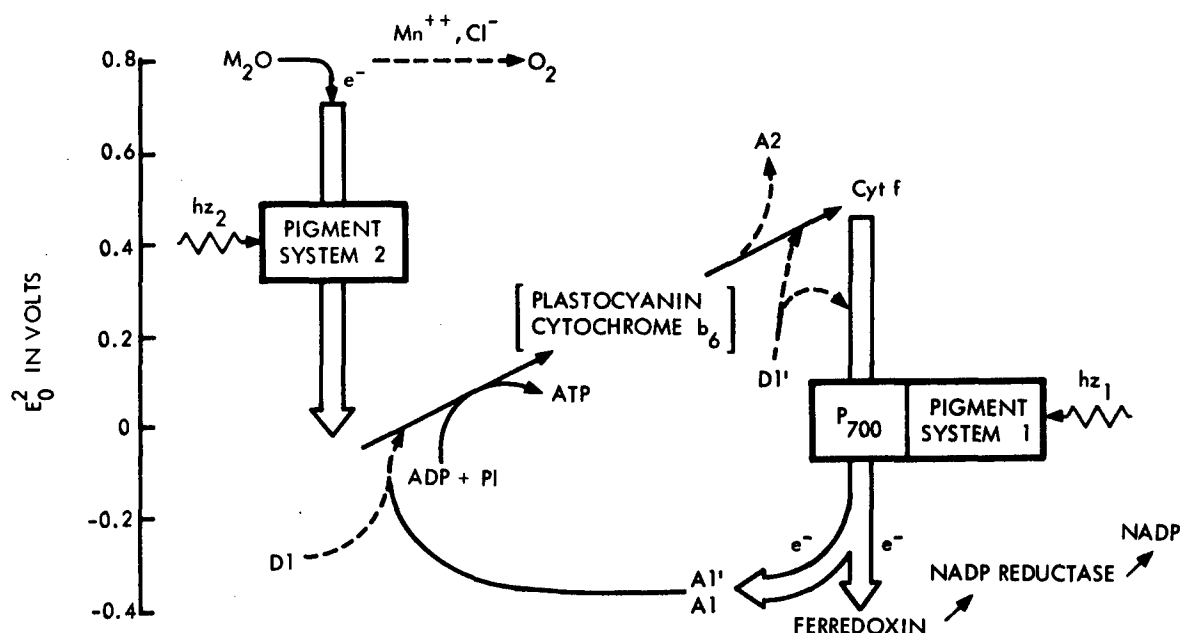


Figure 9. Light-induced electron flow in chloroplasts. All arrows indicate the direction of electron transfer. Heavy arrows indicate the photochemical steps, starting with water oxidation by PS2. The electron acceptor for PS2, plastoquinone (Q), transfers the electron to cytochrome f through an intermediate electron transfer complex (IETC) which contains cytochrome b_6 and plastocyanin in an as yet unknown arrangement. The other photochemical step is catalyzed by PS1, with P700 as the reaction center of the pigment system. Two fates are shown for the transferred electrons, the physiological one leading to NADP reduction and a nonphysiological one leading to reduction of added electron acceptors (A). A1 includes quinones, nitrate, nitrite, indigo, carmine, benzyl viologen, ferricyanide, and manganipyrophosphate. A1' includes those redox agents (DPIP, PMS, FMN, vitamin K_3 , ferredoxin, and quinones having an E_0' below zero volts) which are also capable of donating electrons (after reduction) to the IETC prior to the ATP forming site, and therefore catalyze cyclic electron flow via PS1 with an associated phosphorylation. Donors (D) feed into the system at two sites. D1 includes $PMSH_2$ (below $10^{-5} M$) and $DPIPH_2$ (greater than $10^{-5} M$). D1' includes $PMSH_2$ (greater than $10^{-4} M$), TMPD, and probably $DPIPH_2$ at low concentrations. The D1' donors serve as electron feeders for photoreductions, but no associated phosphorylation is observed. The acceptors listed for A2 include DPIP, ferricyanide, toluylene blue, thionine, and methylene blue. Their reduction via PS2 supports ATP formation, as has been shown for the first two listed. (Vernon and Avron, *Am. Rev. Biochem.* 34, 1965, p. 270).

Clayton's series formulation (1963, 1965):

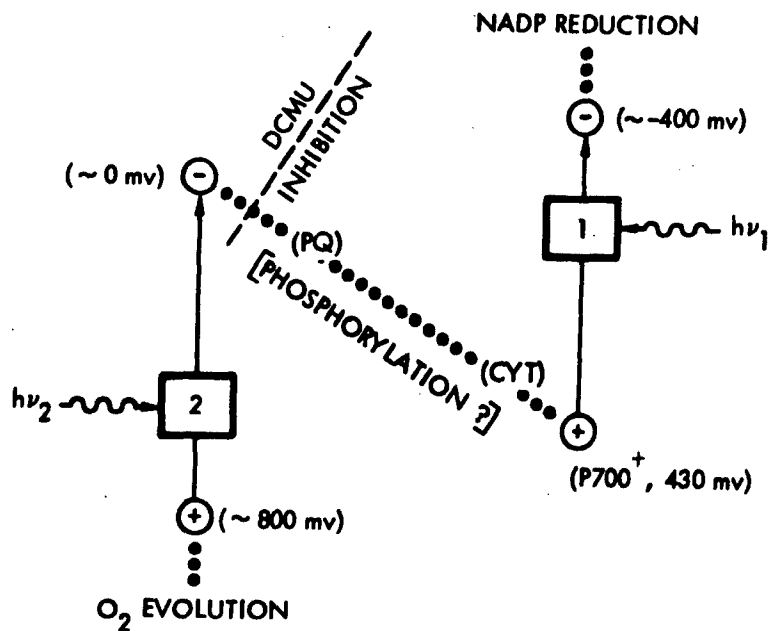


Figure 10. A series formulation for green plant photosynthesis (Clayton, Science 149, 1965, p. 1349)

Another earlier formulation by Hoch and Kok (1961) figure 11, follows:

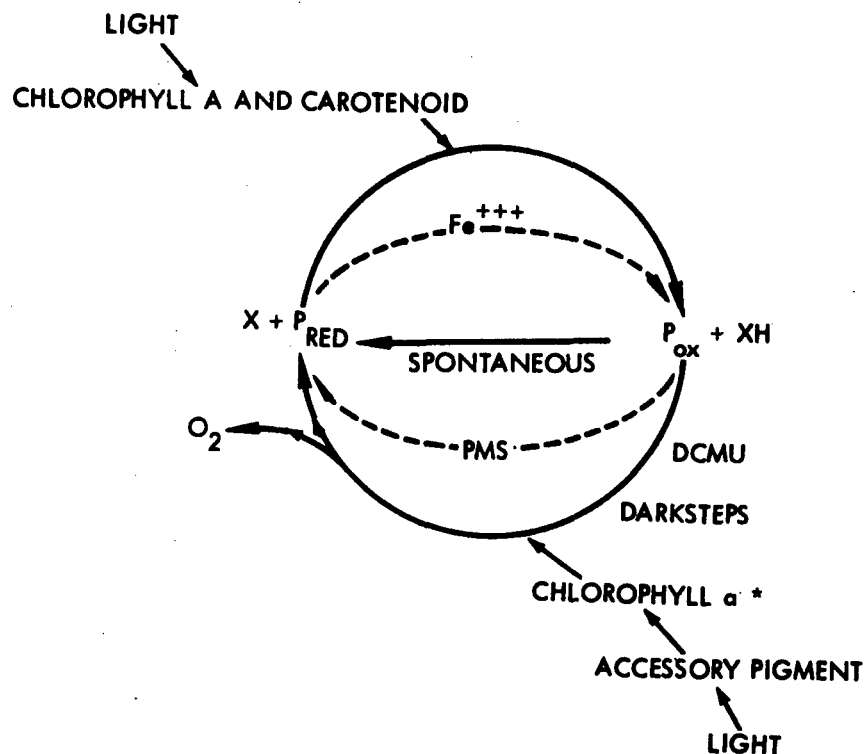


Figure 11. An attempt to correlate observations concerning two photochemical steps in photosynthesis. One is synthesized by chlorophyll-a mainly, the other by accessory pigment associated with another smaller fraction of chlorophyll-a. The two steps oxidize and reduce P700, respectively. A spontaneous backreaction limits the lifetime of the photoproducts from the chlorophyll-a sensitized step. Phenazine methosulfate short-circuits the step driven by chlorophyll-b, whereas ferricyanide is thought to circumvent the chlorophyll-a step. The later assumption definitely seems an over simplification. After Hoch and Kok. (Hoch and Kok, *Am. Rev. Plant Physiol.* 12, 1961, p. 175).

A mechanistic reaction scheme for photoreduction (exclusive of photophosphorylation) in terms of elementary steps has been presented by Robinson (1964), figure 12:

1. Excitation of System II

$$S_{II} + h\nu_{II} = S_{II}^*$$
2. Excitation Transfer to Reaction Center II

$$S_{II}^* + t_{II} = S_{II} + t_{II}^*$$
3. Oxidation of HZ by Electron Transfer

$$HZ + t_{II}^* + Q = HZ^+ + t_{II} + Q^-$$
4. Formation of Z by Proton Transfer

$$HZ^+ + Q^- = Z + HQ$$
5. Oxidation of H_2O and Formation of "Bound" O_2

$$1/2 H_2O + Z = 1/4 O_2(b) + HZ$$
6. Evolution of O_2 Gas

$$1/4 O_2(b) = 1/4 O_2(g)$$
7. Reformation of Q by Dark Back Reaction

$$HQ + U \rightarrow Q + HU$$
8. Excitation of System I

$$S_I + h\nu_I = S_I^*$$
9. Excitation Transfer to Reaction Center I

$$S_I^* + t_I = S_I + t_I^*$$
10. Reduction of X by Electron Transfer

$$t_I^* + X = t_I + X^-$$
11. Oxidation of Cytochrome f

$$t_I^+ + f = t_I + f^+$$
12. Oxidation of HQ by Electron Transfer

$$f^+ + HQ = f + HQ^+$$
13. Reformation of Q by Proton Transfer

$$HQ^+ + X^- = Q + HX$$
14. Reduction of Pyridine Nucleotide

$$HX + PN = X + PNH$$
15. Absorption of CO_2 Gas

$$CO_2(g) = CO_2(b)$$
16. Reduction of "Bound" CO_2 by Calvin-like Cycle

$$1/4 CO_2(b) + PNH = 1/4 [CH_2O] + 1/4 H_2O + PN$$
17. Overall Reaction per Hydrogen Atom Transported

$$1/2 H_2O + 1/4 CO_2(g) = 1/4 [CH_2O] + 1/4 O_2(g) + 1/4 H_2O$$

Figure 12. Reaction scheme for photoreduction (Robinson, Am. Rev. Phys. Chem. 15, 1964, p. 332-3)

The photosynthetic cycle (carbon dioxide enters at the top left), is shown in figure 13, as presented by Calvin in 1962. This is the pathway by which carbon dioxide is incorporated into sugars.

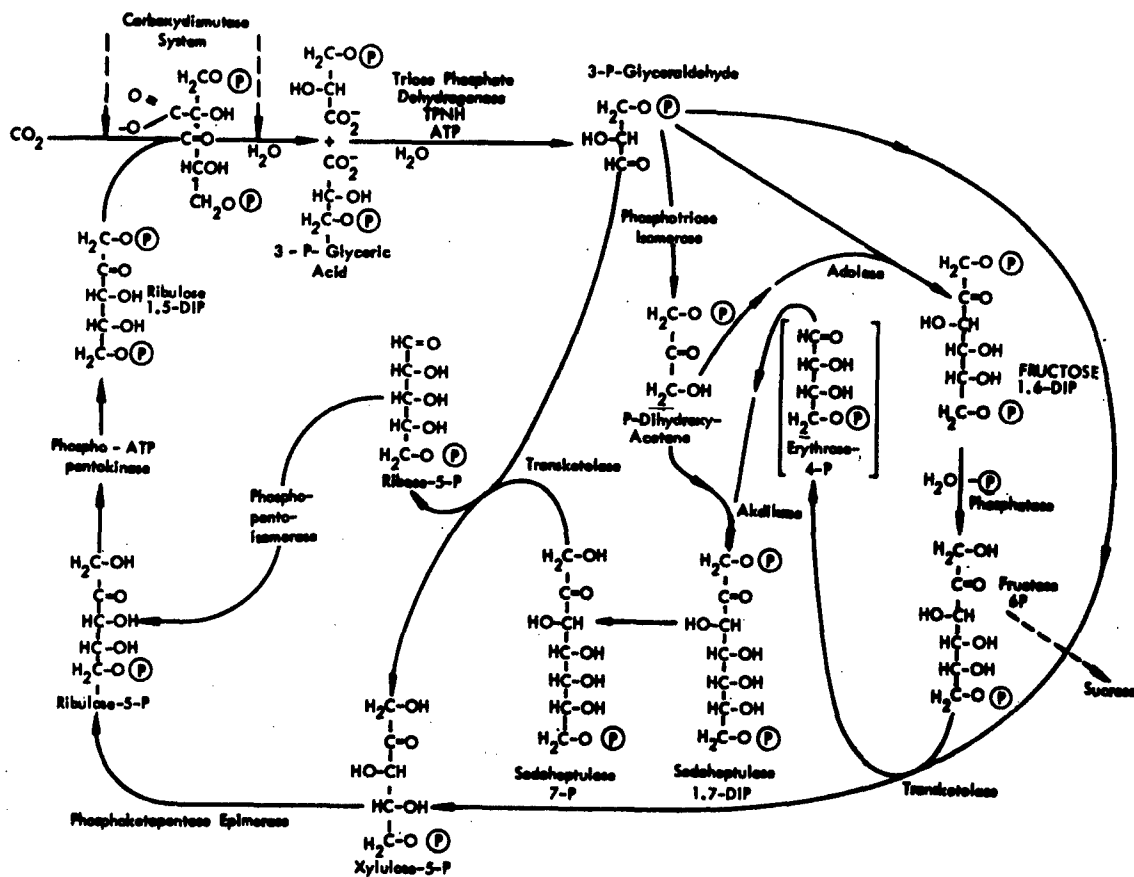


Figure 13, Photosynthetic cycle (Calvin, *Angewandte Chemie, Internat. Edit.* 1, 1962, p. 72)

Another formulation of the photosynthetic cycle by Calvin (1962), showing its relation to succeeding biosynthesis, is illustrated in figure 14:

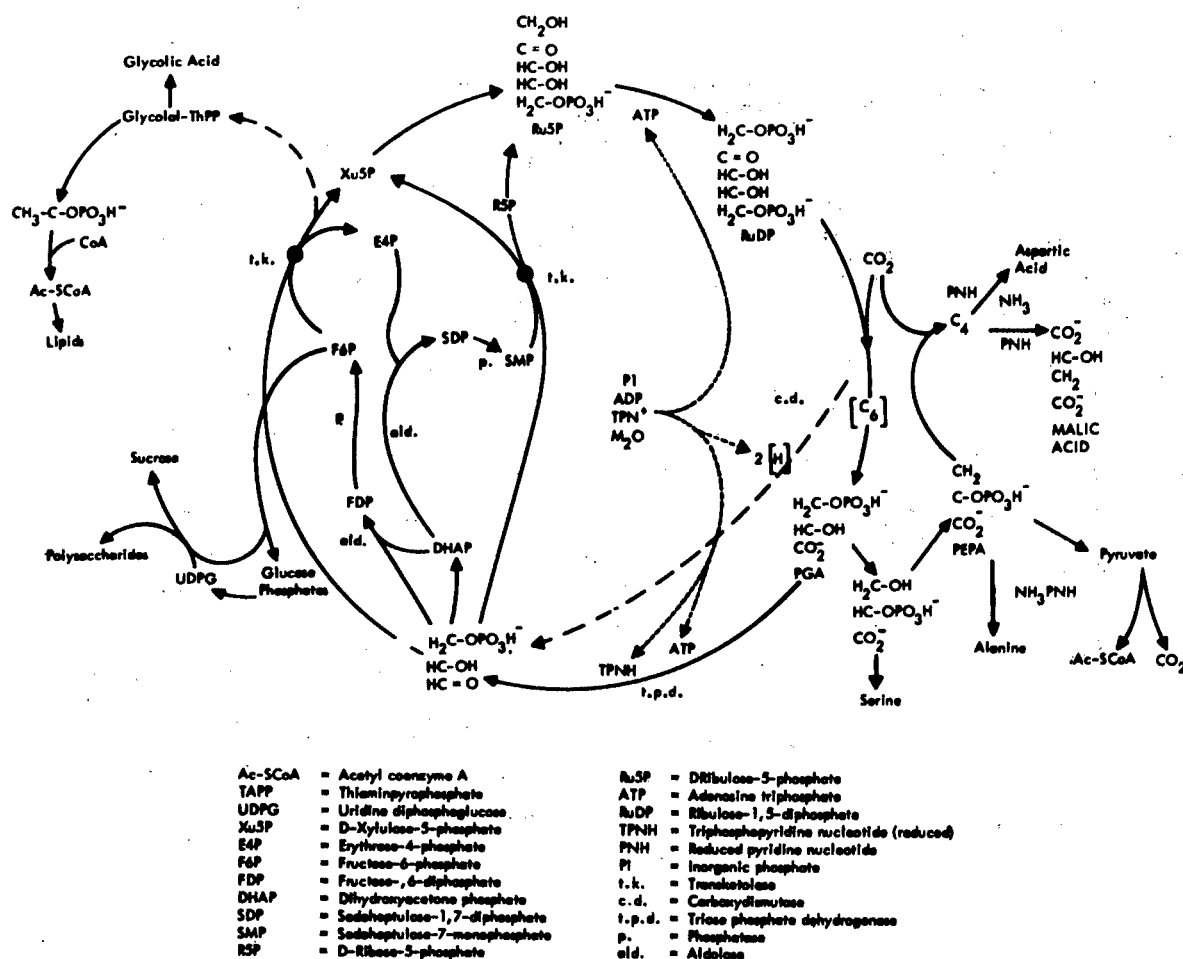


Figure 14. The photosynthetic carbon cycle and its relation to quantum conversion and to succeeding biosynthesis (Calvin, *Angewandte Chemie, Internat. Edit.* 1, 1962, p. 73)

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13. ABSTRACT Where space, weight, and power limitations are of major importance, photosynthesis is an inefficient process for providing the energy for the production of food and oxygen. The assimilation of carbon dioxide into food materials by green plants requires two important factors commonly provided by the photosynthetic process, adenosine triphosphate (ATP) and reduced triphosphopyridine nucleotide (TPNH). We have demonstrated that oxygen as well as enzymatically active TPNH can be generated by an electrochemical system which employs the mediating agents: methyl viologen and ferredoxin-TPN-reductase. This system has been shown to stimulate TPNH-dependent fixation of carbon dioxide. It is recommended that further study be performed to allow greater understanding and definition of the system and its components, and to explore the possibility of producing ATP within the electrochemical cell.			

14.

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